Oxidative Stress in Applied Basic Research and Clinical Practice

Josef Miller
Colleen G. Le Prell
Leonard Rybak *Editors*

Free Radicals in ENT Pathology

Humana Press

Oxidative Stress in Applied Basic Research and Clinical Practice

Editor-in-Chief
Donald Armstrong

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All books in this series illustrate point-of-care testing and critically evaluate the potential of antioxidant supplementation in various medical disorders associated with oxidative stress. Future volumes will be updated as warranted by emerging new technology, or from studies reporting clinical trials.

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Editor-in-Chief

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This book is dedicated to the memory of Gordon Hughes, M.D. (1948–2015). Gordon came to the National Institute on Deafness and Other Communication Disorders in 2008, where he served as the clinical trials coordinator for the NIDCD.

Gordon provided support, advice, and assistance to applicants seeking NIH support for clinical trials in the NIDCD mission areas of hearing, balance, taste, smell, voice, speech, and language. He was a colleague, an advisor, and a source of support for many of the contributors to this edition. His expertise was invaluable, and he will be missed.

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Part I
Introduction

Chapter 1
Introduction: Free Radicals in ENT Pathology

Josef Miller, Colleen G. Le Prell, and Leonard P. Rybak

1.1 Introduction

Basic science research has led to new understanding of the mechanisms of pathology underlying dysfunction in the ear, nose, and throat. This research is defining new interventions that modulate biochemical events leading to pathology and may one day offer new opportunities to prevent and treat impairment. Clearly, many of the advances in our basic understanding and promises for new interventions to prevent and treat pathology are based upon molecular and biochemical studies defining the primary and secondary signaling molecules controlling cellular development and homeostasis, including response to age and stress factors. A key influence increasingly appreciated as contributing to pathology, consequent to many etiological factors, and affecting essentially all tissues and organ systems is the delicate balance that must be maintained in the level of cellular free radicals. While free radicals are essential in the maintenance of normal cellular function, their excess induces cell injury and death. Free radicals directly facilitate biochemical reactions necessary for cell life and participate in cell signaling or "redox signaling,"

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e.g., nitric oxide control of vascular tone. As will be demonstrated in this book, free radical biology is emerging as a defining force determining normal function and, in excess, a common pathway to cell death associated with many etiologies leading to ENT pathology. This book will examine the current state of free radial biology as it impacts on hearing, otology, laryngology, rhinology, and head and neck function. Our intent is to highlight the interrelationship of basic and translational studies in each area, to define the challenges to translation, and to identify the existing basic issues that demand investigation as well as the opportunities for novel intervention to prevent and treat ENT pathology and impairment. In each chapter, or in some cases pairs of chapters, the authors have been charged to include and where possible marry issues of basic research with translational challenges and research, defining the pathway by which new basic insights may lead to interventions to prevent or treat impairment.

Free radicals are highly reactive species (atoms, molecules, and ions) with one or more unpaired electrons in their outer electron shell. While free radicals may be induced by a number of factors and mechanisms, in vivo free radicals are primarily a by-product of the mitochondrial respiratory chain, i.e., normal metabolism of oxygen. The primary role of the mitochondrion or "power plant" of the cell is the phosphorylation of ADP to produce ATP. The process is driven by cellular respiration (oxidization) of succinate (and /or pyruvate) and NADH, products of the citric acid cycle. The process releases electrons which then are passed through a sequential chain of four increasingly electronegative donors-acceptors to oxygen. This process provides the energy to "pump" protons through the inner mitochondrial membrane, creating a proton gradient; and the flow of protons back through that membrane, the ATP synthase complex, drives the synthesis of inorganic phosphate + ADP to ATP.

In a normal, healthy cell, a small percentage of electrons escape the electron transport chain and "leak" directly to oxygen forming reactive oxygen species (ROS), e.g., superoxide anions, hydrogen peroxide, and hydroxyl radicals. And in these normal cells, there are endogenous "antioxidant" enzyme systems to control the excess of free radicals, e.g., thioredoxin, glutathione, superoxide dismutase (SOD), catalase, and melatonin. In addition to endogenous control of antioxidants, a number of exogenous factors, typically consumed as part of our diet, contribute to the control of the number and variety of free radicals available intracellularly by directly scavenging them, e.g., carotenes, ascorbic acid, and α-tocopherol, or by modulating the efficacy of endogenous antioxidant systems, e.g., catalase, glutathione, and SOD. Antioxidants are either hydrophilic, neutralizing radicals in the water compartments of the cell (cytosol), or hydrophobic, reducing radicals in the lipid (membrane) compartments of the cell, and some of these antioxidants function extracellularly. The different sites of action and different mechanisms of antioxidant action contribute significantly to research aimed at defining potential synergistic effects of antioxidants in the control of free radicals.

In normal, healthy cells functioning within a normal operating range of energy demand, the "leak" of electrons that results in the formation of ROS is small, and the free radicals formed can be appropriately controlled by these endogenous

antioxidants.[1] However, in the cell with compromised antioxidant systems, in the cell with age-related injury and DNA damage, or in the cell under extreme environmental stress, the formation of free radicals may exceed the capabilities of the antioxidant systems available and disrupt the delicate balance required for normal homeostasis, resulting in oxidative stress, excessive free radicals, and subsequent cell injury. ROS, specifically superoxide radicals, under enzymatic control, combine with nitric oxide to form reactive nitrogen species (RNS). Excess ROS and RNS directly damage cells and tissues by destruction of cellular and intracellular membranes by lipid peroxidation and by upregulating genes controlling apoptotic pathways and mutagenic damage of DNA.

Etiological factors that can contribute to excess free radical formation affecting cell pathology, including the tissues and systems of ENT, include tobacco smoke,[2] hyper- and hypoxia, UV radiation, intense noise exposure, aminoglycoside antibiotics, chemotherapeutic drugs (e.g., cisplatin), tissue trauma (e.g., implant surgery, with cell death and/or bleeding that lead to superoxides and hydroxides, respectively), age, vascular spasm and reperfusion, viruses and bacteria (leading to cell death and tissue inflammation and secondary to immune responses), inadequate diet (leading to reduced endogenous and exogenous antioxidants), and cardiovascular disease and diabetes (see Chap. 6).

The mechanisms underlying ROS-/RNS-induced pathology are increasingly clear and clearly share some communality across etiology, free radical entity, tissue, and the molecular pathways to cell death. For example, superoxide radicals can interact with nitric oxide to produce a highly toxic molecule, peroxynitrite, that can cause nitration of critical proteins which can lead to cell death in various tissues in the head and neck. This can result in significant morbidity and loss of function in the ear, nose, and throat.

Our initial intent for this book was to provide a comprehensive summary of the work on oxidative stress as a common pathway to pathology across the fields of ENT. The disproportionate research on biology of free radicals focused on the ear and hearing made that impossible. Hence, the majority of the chapters (20 of 24) concern the ear. Three initial basic chapters on the basic biology of free radicals provide the fundamental background to appreciate the more nuanced discussion of ROS/RNS in specific pathologies of the ear that follow: the first provides a broad overview of the basic concept of free radical biology, while the following chapters in this section discuss their role in normal cell function and the increasing evidence for antioxidant interventions to prevent oxidative stress-induced pathology. The second part of the book provides a two-chapter overview (Chaps. 5 and 6) of hearing loss from an epidemiological perspective, assessing the contribution of stress factors related to aging and health to free radical pathology, epidemiological data on hearing loss from environmental factors of noise and heavy metals, and the role of

[1] Although the slow accumulation of free radicals in "normal" cells and tissues is thought to be a significant factor in normal aging and cell death, for individual cells, tissues, and the organism.

[2] As pointed out in Chap. 24, "each puff of a cigarette contains 100 trillion free radicals of varying types."

nutrition on hearing loss. Following sections assess oxidative stress in noise-induced hearing loss, drug-induced hearing loss, age-related hearing loss, and the increasing evidence for a role of free radicals in hereditary hearing loss and antioxidant intervention to prevent and treat such hearing losses. Important concepts within these areas include clinical metrics for protections. There has been little consensus in the field regarding animal models (chinchilla, rat, guinea pig, or mouse), common noise insults (which ranges from 105 to 120 dB SPL across studies), temporary threshold shift (TTS)-inducing noise or permanent threshold shift (PTS)-inducing noise, and whether it is better to measure protection against a small PTS or a large PTS. Other questions may be what day to initiate therapy relative to noise, how long to continue the therapy, and when to assess the final metrics. There has also been little systematic dose-response data collection. We do not know if we are comparing the best (most effective) dose of different drugs or simply two randomly selected doses that happened to yield benefits and were adopted for subsequent studies. These are major issues in the animal literature and are likely to plague human testing as well given the variety of metrics that are emerging for use in studies on prevention of noise-induced hearing loss (NIHL) (Chap. 9) and drug-induced hearing loss (DIHL) (Chap. 12), including conventional air conduction thresholds, extended high-frequency threshold testing, distortion product otoacoustic emissions (DPOAE) tests, auditory brainstem response (ABR) tests, and speech in noise tests. Finally, we include a part on the potential role of surgical trauma on hearing loss in animals and humans and the growing recognition that oxidative stress may play a role in other causes of hearing loss, e.g., Meniere's disease. The final part of the book provides critical chapters describing the role of free radicals on head and neck pathology, primarily cancer related, and their role in sleep apnea and in nasal and paranasal disease.

The final chapter of this book reflects a meeting of all the contributors, culminating in a discussion and "white paper" identifying the challenges to the field and where possible defining the studies and collaborations that may lead to improved understanding of free radical biology in ENT and new interventions to medically treat ENT pathology.

Part II
Basic Science of Free Radical Biology in ENT

Chapter 2
Free Radicals and Oxidative Stress: Basic Concepts and Misconceptions

Jinze Xu and Christiaan Leeuwenburgh

Abbreviations

8-oxodGuo	8-Oxo-7,8-dihydro-2′-deoxyguanosine
8-oxoGuo	8-Oxo-7,8-dihydro-guanosine
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
EC	Epicatechin
ECG	Epicatechin gallate
EGC	Epigallocatechin
EGCG	Epigallocatechin gallate
ELISA	Enzyme-linked immunosorbent assay
ESR	Electron spin resonance
GC/MS	Gas chromatography-mass spectrometry
GPX	Glutathione peroxidase
HPLC	High-performance liquid chromatography
MDA	Malondialdehyde
NADP	Nicotinamide adenine dinucleotide phosphate
RNSs	Reactive nitrogen species
ROSs	Reactive oxygen species
SODs	Superoxide dismutases
TBAR	Thiobarbituric acid reactive substances

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2.1 Overview and Introduction

In the past few decades, remarkable progress has been made toward understanding both the deleterious and beneficial roles of free radicals in mammals. While it is widely recognized that excessive amounts free radicals, including reactive oxygen species (ROSs) and reactive nitrogen species (RNSs), can be harmful and damage cell structures, in strong contrast, at low concentrations they can act as cellular and intracellular signaling molecules that facilitate normal biological processes (Buetler et al. 2004; Bienert and Chaumont 2014; Valko et al. 2007). Truly reactive radicals are those chemical species with one or two highly reactive unpaired valence electrons in open electron shell(s). A large body of evidence suggests that free radicals are linked to the pathogenesis of tumors (Manjunath et al. 2010; Valko et al. 2006; Dwivedi et al. 2008), hearing loss (Yamasoba et al. 2013; Rewerska et al. 2013), tonsillitis (Garca et al. 2013b; Cvetkovic et al. 2009; Yilmaz et al. 2004; Li et al. 2011), allergic rhinitis (Aksoy et al. 2009, 2012), chronic otitis media (Garca et al. 2013a; Baysal et al. 2013; Testa et al. 2012), and rhinosinusitis (Citardi et al. 2006; Uslu et al. 2003) and implies that antioxidant therapy eventually may be employed as a strategy to ameliorate these disorders and diseases (Ding et al. 2013; Tian et al. 2013; Someya et al. 2010). As has been shown recently, however, many antioxidants that are effective in animal models are not effective in human clinical trials.

2.2 Free-Radical Biology

Many sources of free-radical production exist, such as nicotinamide adenine dinucleotide phosphate (NADP) oxidases and mitochondria. The primary function of mitochondria is to produce energy by utilizing oxygen to generate adenosine triphosphate (ATP) for cells. However, mitochondria are also one major source of intracellular ROSs and can generate several reactive radicals and molecules, such as superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^{\bullet}$) (Boveris et al. 1976; Chance et al. 1979; Boveris and Chance 1973; Pollack and Leeuwenburgh 1999). Superoxide anions, but mostly H_2O_2, are diffusible within cells and may act as essential signaling molecules to facilitate normal biological processes and physiology (Buetler et al. 2004; Bienert and Chaumont 2014). Oxidants can react with specific thiol groups on proteins and/or affect a variety of enzymes involved with phosphorylation. In stark contrast, harmful hydroxyl radicals may be formed via Fenton chemistry in the presence of labile transition metals and react immediately and indiscriminately with surrounding biomolecules, including lipids, proteins, and DNA (Koskenkorva-Frank et al. 2013; Halliwell and Gutteridge 1992), and cannot act as specific signaling and/or important redox modification of proteins. Moreover, nitric oxide ($NO^{\bullet}$), which belongs to the RNS family, is involved in several cellular signaling pathways and also is produced by mitochondria (Giulivi et al. 1998).

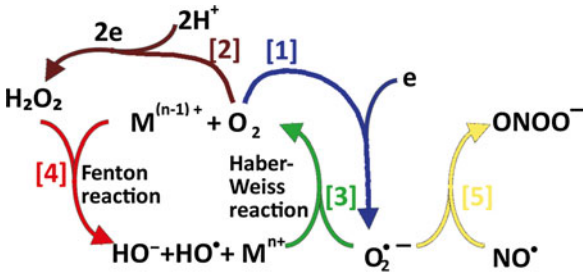

Fig. 2.1 Redox chemistry of free radicals in vivo. Oxygen (O_2) reacts slowly to accept a single electron and produce superoxide ($O_2^{\bullet-}$) in vivo [1], while two electrons can reduce oxygen with the addition of two protons ($2H^+$) to generate hydrogen peroxide (H_2O_2) [2]. Reactive metal ions (M^{n+}), such as ferric iron and cupric ion, play a major role in oxidative stress via Haber–Weiss reaction, which is reduced by $O_2^{\bullet-}$ to reduced form ion ($M^{(n-1)+}$) and oxygen [3]. If H_2O_2 coexists, reduced metal ion will be oxidized to generate a hydroxyl radical ($HO^{\bullet}$) and a hydroxide anion ($HO^{\bullet}$) via Fenton chemistry [4]. In addition, superoxide can react with nitric oxide ($NO^{\bullet}$) to form peroxynitrite ($ONOO^-$), which is a major RNS pathway in vivo and also considered harmful without fulfilling signaling roles

Transition metals, including iron and copper, play an important role in free-radicals-linked damage. Thus, cellular iron, mostly in the ferric form (Fe^{3+}) at physiological pH, can be reduced by $O_2^{\bullet-}$ to ferrous iron (Fe^{2+}) via the Haber–Weiss reaction (Fig. 2.1) (Leeuwenburgh and Heinecke 2001; Xu et al. 2008). While two electrons and two protons coexist, oxygen can be reduced to H_2O_2 reactions, typically accelerated in the presence of enzymes such as glutathione peroxidase (GPX). In the presence of free metals, more highly ROSs can be formed if enzymes like GPX are not readily available. The Fe^{2+} can react with H_2O_2 to generate the extremely reactive $HO^{\bullet}$ by the Fenton reaction. Numerous studies on detecting cellular and mitochondrial labile iron by a wide variety of iron chelators have shown that cells normally maintain a labile iron pool, which exists in a state of dynamic equilibrium between free and ligand iron as well as ferrous and ferric iron (Kozlov et al. 1992; Sohal et al. 1999; Rothman et al. 1992). Certainly in pathophysiological conditions, the quantity of free iron is increased. Iron taken up by eukaryotic cells must reach mitochondria for heme and iron–sulfur cluster biosynthesis (Fig. 2.2) to fulfill one of its biological roles. Upon exposure to oxidants, a minor amount of iron, either loosely bound or poorly liganded to proteins, escapes from storage sites and undergoes the Fenton and Haber–Weiss reactions, particularly within the same compartment (Kakhlon and Cabantchik 2002; Cabantchik et al. 2002; Cantu et al. 2009).

Given the fact that reactive intermediates are transient with extremely short half-lives (10^{-6}–10^{-12} s), it is very difficult to detect them directly in vivo (Leeuwenburgh and Heinecke 2001). A substantial research effort has been directed at exploring end products of oxidative damage to biomolecules, including lipids, proteins, and DNA. The detection of radicals can be performed by direct and indirect measures. Electron spin resonance (ESR) spectroscopy provides a valid tool to investigate free radicals directly by measuring the absorption microwave radiation of unpaired electrons when free radicals are exposed to a strong magnetic field

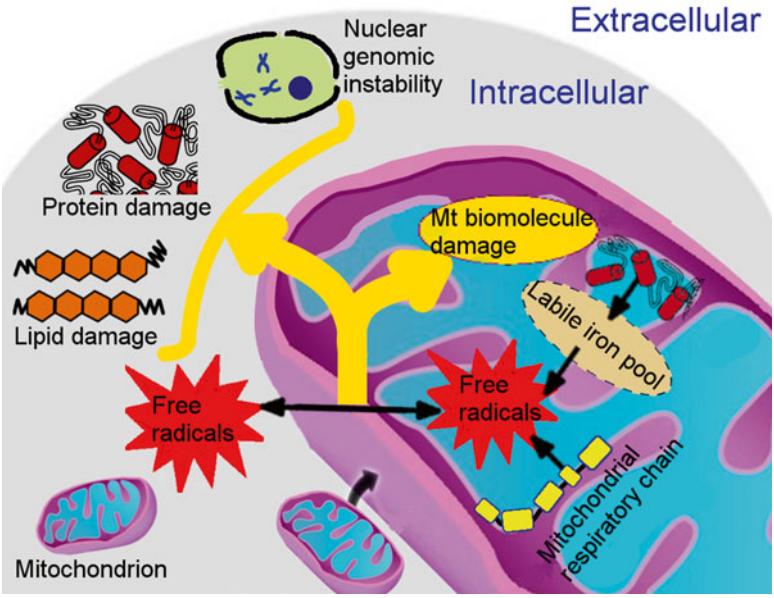

Fig. 2.2 Mitochondrial oxidative stress. The mitochondrial respiratory chain is one major source of free radicals. Mitochondria also express nitric oxide synthase, which produces nitric oxide ($NO^{\bullet}$). Moreover, mitochondria are the major site of heme and iron–sulfur cluster biosynthesis. Iron taken up by eukaryotic cells is transported into the mitochondrial matrix by iron import proteins (e.g., mitoferrin) for mitochondrial iron storage, iron–sulfur cluster biosynthesis, heme metabolism, or other currently unknown pathways. Defects in these processes (e.g., faulty biosynthesis of heme and iron–sulfur cluster) or impaired mitochondrial iron homeostasis contributes to elevated labile iron levels in mitochondria, which has a strong potential to catalyze the reaction of free radicals. Free radicals within mitochondria are directly associated with extensive damage to mitochondrial DNA, which may further lead to mitochondrial dysfunction. More stable ROSs can reach other intracellular areas, resulting in cytosolic damage of other organelles

(Davies et al. 1982). Lai et al. successfully used ESR to demonstrate that the superoxide dismutases (SODs) predominantly localized in epithelial cells of rat nasal mucosa scavenge microenvironmental $O_2^{\bullet-}$ radicals (Lai et al. 1997). Moreover, spin trap compounds generally are necessary to detect short-lived radicals. A spin trap reagent can react with the radical to form a spin adduct that is relatively stable and can be detected with ESR spectroscopy. However, in biological systems where multiple radicals or oxidants are present, many may form the same radical adduct with a spin trap reagent. Thus, use of spin trap compounds with ESR spectroscopy may raise an issue of their selectivity and specificity.

Except for biological fluids, radical detection protocols are based mostly on cell-disruptive methods, which inevitably alter the equilibrium between radical species as well as between radicals and various cellular components. Current non-disruptive techniques for detecting radicals and labile ions in vivo dramatically rely on the application of fluorescent probes, which comprise a fluorescent group

coupled with a high-affinity chelator (Cabantchik et al. 2002; Kakhlon and Cabantchik 2002). Moreover, probes must be lipophilic, membrane-permeable, and able to compete with endogenous ligands and polypeptides (Petrat et al. 2002; Rauen et al. 2007). For instance, labile iron in cells and tissues can be visualized by fluorescent probes, such as Phen Green SK and calcein. A recent study by Cantu et al. employing an iron indicator rhodamine B 4-[(1,10-phenanthrolin-5-yl)aminocarbonyl]benzyl ester has shown that mitochondrial aconitase releases labile iron under oxidative stress in primary ventral mesencephalic cells (Cantu et al. 2009). This event was followed by mitochondrial dysfunction and cell death. Furthermore, this study's observation that removal of H_2O_2 and labile iron, using catalase and an iron chelator (*N*,*N*′-bis(2-hydroxybenzyl)ethylenediamine-*N*,*N*′-diacetic acid), mitigated cell death strongly suggests that H_2O_2 and labile iron mediate oxidative damage and cell death.

More indirectly, radical activity—damage—can be detected on a variety of biomolecular fingerprints. The polyunsaturated acids of the fatty acid membrane contain more than one double bond in their alkyl chain, which are extremely vulnerable to ROSs. The end products of oxidant reactions with these vulnerable structures (lipid peroxidation reactions) include alkanes, isoprostanes, malondialdehyde (MDA), and isoketals. The thiobarbituric acid reactive substance (TBARS) assay is a typical nonspecific test that measures lipid peroxidation by detecting MDA levels. Thiobarbituric acid reacts with MDA in acidic condition to form a pink pigment, which has an absorption maximum of 532 nm. Absorbance changes at 532 nm are linearly correlated with chromogen concentration, and the amount of chromogen can be quantified by spectrophotometer (Buege and Aust 1978). In addition, gas chromatography/mass spectrometry (GC/MS) is a powerful technique for measuring MDA or other more robust lipid peroxidation markers, e.g., F_2-isoprostane, which present in all normal biological fluids and tissues (Morrow and Roberts 1997).

Reactive radicals' attacks can modify amino acid residues in the proteins. Reactive hydroxyl radical ($HO^{\bullet}$) converts the amino acid phenylalanine to ortho-, meta-, and para-tyrosine. Peroxynitrite ($ONOO^{-}$), which is formed by the reaction of $O_2^{\bullet}$ with $NO^{\bullet}$, can mediate the nitration of the ortho position of tyrosine residue to form 3-nitrotyrosine. Trace amounts of tyrosine isomers and 3-nitrotyrosine can be detected by GC/MS and high-performance liquid chromatography (HPLC).

Oxidation of specific amino acid residues (lysine, arginine, proline, and threonine) of proteins forms carbonyl species (aldehydes and ketones). Lipid oxidation with the covalent adduction of lipid aldehydes and sugar reduction also contribute to the increase in carbonyl levels (Dalle-Donne et al. 2003). Hence, the carbonyl assay indicates not only protein oxidation but also lipid peroxidation products. Carbonyl levels, however, can be quantified by various techniques, including spectrophotometry, Western blot immunoassay, enzyme-linked immunosorbent assay (ELISA), and GC/MS, which are accessible and can be performed in any biomedical research laboratories. Thus, carbonyl assays are widely accepted for evaluating protein oxidation, with the caveat that additional analysis is required to exclude the carbonyl groups from lipids and sugar.

Free-radical-induced damage to DNA and RNA yields numerous products. The most popular biomarkers of DNA and RNA are guanine base oxidation products, 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo) and 8-oxo-7,8-dihydroguanosine (8-oxoGuo), respectively, which can be quantified simultaneously using HPLC with an electrochemical detector (Hofer et al. 2006).

2.3 Select Misconceptions in Free-Radical Biology

As briefly discussed earlier, under normal (non-pathophysiological) conditions, a minor amount of free radicals within cells is essential for cellular and intracellular signaling systems. In addition, cells are well protected against oxidative damage by delicate and sophisticated defense systems. The biological activity of free radicals is maintained in balance by endogenous antioxidants and antioxidant enzymes. A sudden acceleration in the production of free radicals (i.e., ischemia reperfusion) or a chronic deficiency in the antioxidant defense system may alter this balance, which leads to free-radical-induced damage to biomolecules, and may be further involved in the pathogenesis of various disorders and diseases (Valko et al. 2007). Levels of beta-carotene, retinol, vitamin E, and vitamin C, for example, were observed to be significantly decreased in children with acute otitis media and tonsillitis (Cemek et al. 2005). Manjunath et al. reported that serum MDA levels were significantly elevated and that ferric reducing antioxidant power was dramatically decreased in patients with laryngeal and hypopharyngeal cancer (Manjunath et al. 2010). Moreover, Aksoy et al. revealed an association of patients with allergic rhinitis to increased serum protein oxidation levels (Aksoy et al. 2009, 2012). Other findings add to the evidence that elevated serum oxidant levels and declined antioxidant status levels were observed in patients with chronic long-term otitis media (Garca et al. 2013a; Baysal et al. 2013; Testa et al. 2012).

Current understanding of the involvement of free radicals in pathology suggests that antioxidant therapy through attenuating free radicals may be important to optimize treatment (Ding et al. 2013; Tian et al. 2013; Someya et al. 2010; Cemek et al. 2005). Most studies using animal models indicate the benefits of antioxidants in the prevention of acute oxidative conditions and diseases. Bonabi et al., for instance, reported that resveratrol had a significant protective effect against gentamicin-induced toxicity in cultured organ of Corti explants from newborn Sprague–Dawley rats (Bonabi et al. 2008). A recent study on noise-induced hearing loss in Fischer 344 rats showed that resveratrol dramatically reduced noise-induced cyclooxygenase-2 expression and ROS formation (Seidman et al. 2013). Moreover, the antioxidant N-acetylcysteine combined with disodium 2,4-disulfophenyl-N-tert-butylnitrone as a therapeutic intervention for noise-induced hearing loss in rats was found to be effective in reducing both temporary and permanent threshold shifts across all frequencies (2–16 kHz) in auditory brainstem response tests (Lu et al. 2014). A clinical study of radix astragali, a natural antioxidant herb, indicated its beneficial effect in recovering hearing in patients with sudden deafness (Xiong et al. 2012).

For decades, a substantial research effort has focused on coenzyme Q10 as a therapeutic approach to ENT pathology. A study in a guinea pig model of noise-induced hearing loss by Fetoni et al. demonstrated that coenzyme Q10 decreased active caspase-3 expression and the number of apoptotic cells and improved outer hair cell survival (Fetoni et al. 2009). The same research group also found that coenzyme Q10 significantly mitigated noise-induced auditory function and hearing loss in rats (Fetoni et al. 2012). (Other studies demonstrating the efficacy of antioxidant intervention in the prevention of noise- and drug-induced and age-related hearing loss can be found in Chaps. 10, 11, 12, 13, 14, 15 and 16 of this book.)

Though scientists have made considerable progress in support of antioxidant supplements in pathology, human research findings remain controversial and inconsistent, particularly in epidemiological and clinical studies. A prospective study of vitamin intake and the risk of hearing loss in men aged 40–74 years by Shargorodsky et al. found a lack of correlation between higher intake of vitamins C, E, beta-carotene, or B12 and the risk of hearing loss (Shargorodsky et al. 2010). Consistently, 5-year longitudinal analyses on the link between diet quality and hearing loss in the elderly did not provide robust efficacy data (Gopinath et al. 2014). In a previous study on the glutathione supplement in 30 patients with hearing loss, the treatment failed to modify significantly the progression of hearing loss (Sataloff et al. 2010). On the other hand, studies by Choi et al. and Spankovich et al. both demonstrate relations between use of antioxidant supplements and reduced risk of age-related hearing loss in elderly humans. Despite the differences in the protocols, the conflicting study observations may stem from several factors or misconceptions involved in free-radical biology.

2.4 Antioxidants May Blunt Benefits that Depend on ROS Signaling

Previous research has demonstrated that ROSs actively participate in a variety of biological signaling processes. Appropriate increases in oxidative stress as effector molecules can trigger stress-inducible genes, which participate in cellular repair. Thus, modest stimulation of ROSs is beneficial. Indeed, caloric restriction, with an associate increase in ROS formation, has been shown to extend life span in *C. elegans* (Schulz et al. 2007). Exposure to H_2O_2 can also activate transcription of the DNA-damage-response gene and induce DNA repair in HeLa cells (Furukawa-Hibi et al. 2002). Another example of hormetic increases in ROSs is moderate aerobic exercise, which exerts beneficial health effects mediated through direct ROS increases (Mendelsohn and Larrick 2013). In general, acute exercise increases oxidant levels and oxidative stress in untrained animals, but long-term exercise may counter this effect by increasing the activity of antioxidant enzymes and reducing oxidant production. These defenses may be critical for preventing chronic oxidative muscle damage during exercise and even at rest. We have assayed the activities of antioxidant enzymes in rat skeletal muscle samples following exercise training.

In most of our studies, the activities of two major antioxidant enzymes, mitochondrial SOD and cytosolic glutathione peroxidase, were significantly higher in exercising animals than in sedentary animals. There was little difference in catalase and cytosolic SOD activities (Leeuwenburgh and Heinecke 2001). This suggests that mitochondria are a source of constant free-radical production, which may cause beneficial adaptations. We also consistently detect lower levels of oxidative stress markers following the exercise training. These results indicate that increased activities of GPX and mitochondrial SOD might account in part for the decline in oxidative damage in exercise-trained animals. What may be surprising is the fact that increased mitochondrial radical production with moderate bouts of exercise can provide long-term beneficial adaptations. During the short burst of exercise, metabolism is characterized by a high rate of adenosine diphosphate (ADP) formation because ATP breakdown increases with workload. High ADP levels activate oxidative phosphorylation, thereby dramatically increasing the levels of electron flow in the respiratory complexes. This flow of electrons toward oxygen reduces the amount of superoxide production but appears sufficient to increase the induction of major antioxidant enzymes GPX and SOD. While some studies now show that antioxidant supplementation can blunt the normally required free-radical production of SOD and GPX induction, more human research is needed. Together these results suggest that antioxidant supplements can blunt the beneficial effects in humans by counteracting with transient hormetic responses in ROSs.

2.5 Many Factors Can Impact the Bioavailability of Antioxidants

In laboratory animal studies, diet compositions are precisely controlled and animals are genetically homogeneous. With heterogeneous backgrounds and different dietary habits in human research participants, individual differences in dietary nutrients, plasma cholesterol profile, gender, and age could have major influences on the bioavailability of antioxidant supplementation.

Antioxidant therapies generally are either hydrophilic or hydrophobic compounds. The use of hydrophobic antioxidants in humans usually proves unsatisfactory, most likely because of their relatively low oral bioavailability and complex absorption process. Coenzyme Q10, for example, is relatively insoluble in aqueous solutions, has a relatively high molecular weight, and has a limited solubility in lipids, which directly results in about 2–3 % absorption when orally administered to rats. Other factors also impact plasma coenzyme Q10 levels after oral administration. There is evidence showing that dietary fat enhances coenzyme Q10 absorption. On the contrary, uptake of vitamin E together with coenzyme Q10 intervened and decreased coenzyme Q10 absorption. These brief examples demonstrate the many effects of administrating a single antioxidant in research studies, thus making interpretation complicated.

2.6 Antioxidants May Act as Prooxidants

Antioxidants are highly reactive and prone to be oxidized. Many factors can influence their stability and trigger oxidation reaction, including light, temperature, pH, and metallic ions.

Previous research in vitro has demonstrated the beneficial outcomes of tea polyphenols, which are potent hydrophilic antioxidants and can scavenge ROSs, RNSs, and redox-active transition metal ions. Epigallocatechin gallate (EGCG) is the primary bioactive compound in green tea, followed by epigallocatechin (EGC), epicatechin gallate (ECG), and epicatechin (EC). Green tea polyphenols are stable in acidic solutions with pH values ranging from 1.0 to 6.0. EGCG with a galloyl moiety is the most vulnerable to pH among them, which results in its instability at physiological pH 7.4. Much attention has therefore focused on the potential prooxidative role of EGCG, particularly in a number of in vitro assays. A decade ago, several lines of evidence suggested EGCG was involved in inducing H_2O_2 generation and DNA damage in HL-60 cells (Furukawa et al. 2003; Oikawa et al. 2003). Suh et al. reported similar results, in which EGCG was positively associated with apoptotic cell death and H_2O_2 production in a dose-dependent manner in HIT-T15 pancreatic beta cells (Suh et al. 2010). In the same study, the observation that removing iron using iron chelators inhibited ROS formation strongly suggests that transition metals are essential catalysts during the process. In this case, some oxidants can be used to kill harmful (cancer) cells but may also damage noncancerous cells in the body. Because there is substantial evidence that selected antioxidants also exhibit prooxidant activity, it will be essential to study antioxidant compounds in various cell types and under physiological conditions.

2.7 Conclusions

Free radicals have been shown to be involved in various pathology conditions. An increase in research on antioxidants demonstrates that their ability to scavenge free-radical species and labile transition metals could provide therapeutic approaches to treating free-radical-induced conditions and diseases. Some studies in animal models and human subjects, however, have been less conclusive. This raises the concern that the complexities of free-radical biology and antioxidant treatments as well as practical variations of human participants may make it impossible to study and obtain interpretable results on the efficacy of single antioxidant compounds. Physical exercise and a healthy diet that includes a high intake of natural complex antioxidant mixtures, for example, may influence the effects of antioxidant supplementation by (1) interfering with metabolism and bioavailability, (2) failing to meet physiological levels within the cell at critical sites and exacting oxidant production sources, or (3) blunting benefits at other locations that depend on ROS signaling. Moreover, antioxidants are vulnerable to rapid changes in oxygenation (ischemia/reperfusion), light, temperature, and, most importantly, redox-active transition

metals, which could act as prooxidants under certain conditions, both in vitro and in vivo. These issues do not mitigate against the use of supplemental antioxidants to prevent or treat pathologies for which there is a rich basic science database indicating an important role of free radicals in the pathology, but it surely makes it difficult to argue for such use on the basis of empirical human findings.

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Chapter 3
A Question of Balance: Free Radicals and Cochlear Homeostasis

Kevin K. Ohlemiller

3.1 Introduction

The hearing research landscape features a host of animal studies aimed at reducing cochlear injury caused by oxidative and nitrosative stress. Most of these report only partial success and reveal limitations of a naïve "less is better" philosophy toward oxidative intermediates. Early exuberance has given way to a more sober realization that few reactions can be tweaked without consequences for many other critical reactions and that the potential set of reactions to be considered varies cell by cell and compartment by compartment. In this chapter, we explore how different cochlear cells and structures may have different effectors of oxidative stress, yet how they may depend on each other in establishing the overall effects of oxidative stress on hearing.

3.2 Oxidative and Nitrosative Homeostatic Balance

Oxidation reactions involve loss of electrons. In biological systems, the most common oxidation reaction is the addition of oxygen to organic molecules (carbon + hydrogen), which (perhaps in the schoolyard sense) then get to "share" their electrons with oxygen. The notion of "redox" (reduction–oxidation) simply acknowledges that every oxidation has an electron "loser" (the molecule or atom that loses its share of electrons) and a "winner" (the moiety that gains a share of electrons), which is said to be "reduced." Most crucially for our purposes, oxidation

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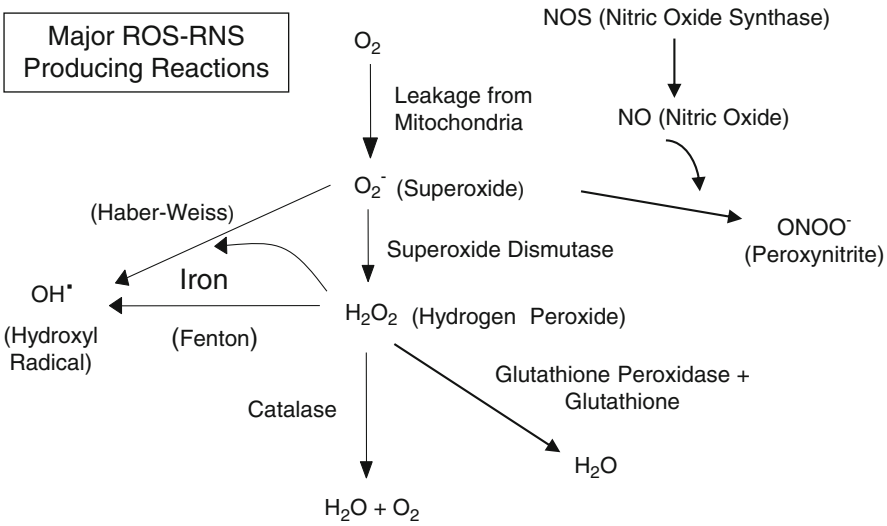

Fig. 3.1 "Core" reactions that produce or remove reactive oxygen species

renders large organic molecules—proteins, lipids, and DNA—less functional. Oxidative reactions associated with cellular injury include those giving rise to highly reactive oxygen-containing radicals (superoxide, hydroxyl, peroxyl) and merely "reactive" oxygen-bearing species (peroxide and hypochlorous acid), which may either act as oxidants or convert to radicals (Berg et al. 2004; Linnane et al. 2007; Ciuman 2009; Poirrier et al. 2010). All these are collectively termed reactive oxygen species (ROS).

Instead of acting directly as an oxidant, superoxide can react with nitric oxide (NO) to form peroxynitrite, a potentially more reactive oxidant (Radi 2013) (Fig. 3.1). Cellular variation in NO production, in turn, depends on the distribution of nitric oxide synthase (NOS) isoforms. Nitrogen-bearing oxidants are collectively termed reactive nitrogen species (RNS), although peroxynitrite is often the only one considered. The "core" ROS- and RNS-related reactions and mediating enzymes are depicted in Fig. 3.1 (see also Chap. 2). Superoxide may also convert spontaneously or enzymatically to either peroxide (H_2O_2) or peroxynitrite. Since superoxide lies in both cascades, there is overlap of the processes that generate ROS and RNS. Enzymatic conversion to peroxide is mediated by one of several isoforms of superoxide dismutase (SOD). Peroxide in turn is enzymatically converted to water or may react in the presence of iron to form a hydroxyl radical. The innocuous conversion to water is mediated by catalase, a peroxisomal enzyme, or by one of several isoforms of glutathione peroxidase (GPx). GPx operates by transferring electrons from cofactor glutathione, a small molecule antioxidant that may be present in cells in concentrations as high as 5.0 mM. The critical role of catalase and GPx is generally thought to be the minimization of a highly toxic hydroxyl radical.

There remains debate among serious chemists (which this author makes no claim to be) as to which of the reactions in Fig. 3.1 are truly pathogenic and exactly what each of the "core" antioxidants evolved to accomplish (Linnane et al. 2007; Bartosz 2009; Gutteridge and Halliwell 2010; Sohal and Orr 2012). The fact that many therapeutics targeting ROS or RNS generally confer hearing protection (see below) certainly suggests that some reactions in Fig. 3.1 are best minimized. However, the mixed success record of such therapeutics indicates that Fig. 3.1 is missing too many elements, or too much spatial variation, to serve as a template for therapies. One entire class of peroxide-removing enzymes, the peroxiredoxins, may play a larger role than is generally credited (Rhee et al. 2005). As we noted, NO production is hugely dependent on the distribution of NOS isoforms, which vary by cochlear cell type. This may help explain an extremely confusing literature regarding the helpful-versus-harmful status of NO.

3.2.1 The "Purpose" of ROS and RNS Generation and Requirement for Balance

Without redox reactions, there would be no interesting or vital organic chemistry, so it is not as if life could be founded on some safer—less mischievous—set of reactions. Cells must be able to break down complex organic molecules and to kill invading pathogens. Both sets of reactions are oxidative. Eukaryotic cells produce superoxide as part of the mitochondrial respiratory chain. Environmental stressors, such as heat, osmotic stress, or pH imbalance, can enhance net superoxide production by mitochondria. Superoxide generation by phagocytes also occurs through the actions of a class of enzymes termed NADPH oxidases (NOX), particularly NOX2 (Bylund et al. 2010). However, nearly all cells produce one or more NOX isoforms, partly because most cell types retain some intrinsic ability to kill pathogens. NOX3 is especially highly expressed in the cochlea, where it may also participate in the formation of otoconia (Banfi et al. 2004). For reasons that are unclear, it is also highly expressed in hair cells and spiral ganglion cells, albeit minimally in the cochlear lateral wall. Under conditions of stress (e.g., noise and ototoxic exposure), NOX3 has been suggested to be the single largest producer of superoxide.

In addition to NADPH oxidases, cells produce a host of functionally similar enzymes residing in the cell membrane whose primary function may be generation of superoxide and peroxide to serve as messengers. Some have argued that *most* peroxide production is adaptive (Rhee 2006; Linnane et al. 2007; Sohal and Orr 2012). According to one line of logic (Bartosz 2009), any moiety that is created in an enzyme-catalyzed reaction serves a purpose. When the product is a potential oxidant, it will possess a narrow non-pathological concentration range. Moreover, the enzymes that make the product must be balanced by other enzymes that remove it from the same compartment (Rhee 2006). Many transcription factors and other mediators that drive cell processes in non-stress contexts (e.g., division, migration, secretion, and contraction) are redox-sensitive, including AMP-activated protein kinase (AMPK), platelet-derived growth factor (PDGF), p38 MAP kinase, c-Jun

N-terminal kinase (JNK), and nuclear factor kappaB (NFκB) (Veal et al. 2007; Bartosz 2009). In such cases, transcriptional activation relies on reversible modifications to transcription factors, cofactors, and binding sites. Under conditions of overt stress (heat, osmotic stress, pH change, shear stress, hypoxia, hyperoxia, infection), oxidant concentrations may approach the pathological range and engage mediators of antioxidant defenses and repair-versus-die "decisions." These overlap with the previous set and include hypoxia-inducible factor 1α (Hif1α), Nrf-2, Atf1, JNK, and NFκB. Injury to excitable cells (as in the brain and cochlea) can be modulated by altering ion conductances, particular to K^+ and Ca^{++}. Thus it is no surprise that voltage-activated and gap junctional conductances are readily altered by oxidation, often (not in all cases) in such a way as to dampen activation (Bartosz 2009).

In the inner ear, peroxide production by SOD and removal by catalase and GPx appear to operate in critical balance. Targeted inactivation of GPx1 and SOD1 in mice was found to accelerate age-associated hearing loss and exacerbate the effects of noise (McFadden et al. 1999a, b, 2001; Ohlemiller et al. 1999, 2000), but overexpression of SOD1 was found to be harmful to the cochlea (Coling et al. 2003). How toxic is peroxide to the inner ear? Experiments attempting to demonstrate peroxide toxicity in the cochlea (Clerici et al. 1995; Clerici and Yang 1996) and other tissues (Linnane et al. 2007; Sohal and Orr 2012) have used nonphysiologically relevant high levels, so that these may not be very informative. Purely pathologic functions of peroxide may result from its conversion to hydroxyl radical through reactions with iron via Fenton/Haber–Weiss chemistry (Fig. 3.1). Hydroxyl radical seems to have no adaptive role in cell physiology. However, it is so reactive—reacting with the first molecule it encounters—that some have expressed doubts that it is truly biologically relevant (Linnane et al. 2007).

Knockout mouse models generally support the notion that impairment of the "core" antioxidants of Fig. 3.1 can produce individuals that are susceptible to environmental stress (Yoshida et al. 1997; Lei 2001; Ho et al. 2004). This principle appears to extend to acquired hearing loss in humans. Certain single nucleotide polymorphism (SNP) variants of genes encoding a number of redox-related enzymes, including catalase, SOD2, paraoxonase-2 (PON2), and glutathione-S-transferase M1 (GSTM1), have been implicated in decreased resistance to permanent threshold shifts (PTS), particularly in the context of habitual noise exposure (Sliwinska-Kowalska and Pawelczyk 2013) (see also Chaps. 8 and 14). GSTM1 genotypes associated with increased hearing loss in humans included homozygous nulls—that is, individuals who do not produce this enzyme at all. Such results are relevant to how we conceptualize and search for "pro-PTS" alleles. It might be argued that testing candidate genes in homozygous null (knockout) animals represents an unrealistic and biased way to study such genes, since alleles contributing to PTS in humans will typically not be nulls *nor* will they be inherited in a homozygous state. However, SNP variants are limited to just four allele types (since there are only four possible bases), of which subsets will be enriched in certain populations, and thus are likely to be inherited in homozygous form. The GSTM1 results further argue that some naturally occurring variants may, in fact, be null alleles. Most of the current evidence for pro-PTS alleles in humans is tentative, as most

studies used small samples and very few have been replicated. Part of the difficulty is that relevant human gene variants are likely to adhere to "common variant" notions, whereby alleles of many genes that are common across populations each contribute only a small amount of risk.

The set of reactions falling under the rubric of nitrosative stress begins with the generation of nitric oxide (Berg et al. 2004; Heinrich and Helling 2012). Through reaction with superoxide, NO promotes the production of peroxynitrite (Radi 2013). Nevertheless, adaptive functions have been proposed for both NO and peroxynitrite. NO is produced by distinct synthase isoforms whereby it supports neuronal synaptic function (through the activity of neuronal NOS, nNOS) and promotes increased numbers of mitochondria and vasodilation (through endothelial NOS, eNOS). Maladaptive generation of NO is driven most often by stress-related activation of inducible NOS (iNOS), which is minimally detectable in the cochlea under normal conditions (Shi et al. 2003), but can produce much higher amounts of NO than the other isoforms. Notably, the primary activity of iNOS in the body occurs in macrophages in response to pathogens or exposure to lipopolysaccharide (LPS, a component of bacterial cell walls), so that iNOS-related nitrosative stress represents an aspect of inflammation. Within the cochlea, LPS, noise, and ototoxins can all upregulate iNOS (Heinrich and Helling 2012).

Peroxynitrite, the single major RNS in much of the literature, is moderately membrane permeant and may diffuse several cell diameters before reacting with proteins, lipids, and nucleic acids (Radi 2013). The reaction of NO with superoxide to form peroxynitrite is much faster than the reaction of superoxide with SOD. Hence, local production of peroxynitrite is highly sensitive to the joint concentration of superoxide and NO.

3.2.2 *What Evolutionary Forces Shaped the Stress Responses of the Inner Ear?*

It is tempting to think of evolution as a pure "optimizer" (through selective pressure) of how cells and organisms respond to their environment. It is questionable, however, how strongly selective pressures could have acted to optimize responses of cochlear cells to industrial age noise or ototoxins (for discussion, see Kirk and Smith 2003). It makes sense that mechanisms (e.g., cochlear efferents) evolved to impart gain control and to optimize signal detection in noise and that these may be co-opted to modulate noise injury. But apparent gaps in regulatory systems, and some clearly *maladaptive* responses of the cochlea to stress, conjure a vision of evolution more like a toddler with a jackhammer than a skilled mechanic. Innate shortcomings that now leave researchers trying to play the role of optimizer are likely the result of selection for one feature working at cross-purposes to other necessary features that may not be obvious. For example, hair cell self-repair seems limited, yet hair cells convinced not to die by anti-apoptotic treatments show surprising capacity for self-repair (Pirvola et al. 2000). Why is this not the natural

response? A variety of preconditioning paradigms are highly effective in protecting hair cells from noise (e.g., Canlon 1997; Yoshida et al. 1999; Yoshida and Liberman 2000; Gagnon et al. 2007; Fernandez et al. 2010), yet there must be a downside to maintaining the preconditioned state or it would always be engaged. A frequent evolutionary driver for preconditioning was probably bacterially and virally derived endotoxin (Tsai et al. 2004). This would help explain why the response of the inner ear to virtually any stressor we invoke somewhat resembles a response to pathogens. In both noise- and ototoxin-exposed cochleas, hair cells, supporting cells, and cells of the lateral wall secrete a host of cytokines. The actions of these are neither unambiguously good nor bad. Chief among their actions is the apparent recruitment of inflammatory cells from the vessels of the spiral ligament (Hirose et al. 2005; Tornabene et al. 2006). Following noise, LPS, or ototoxins, multiple distinct types of macrophages (as determined by their surface receptors) invade the ligament and the lining of the scala tympani. By and large, they seem to migrate to sites of cell loss (spiral ligament and limbus) and may primarily simply be clearing debris. Yet they rarely invade the organ of Corti, even when there is extensive hair cell loss. Within the organ of Corti, their debris-clearing role may be supplanted by resident macrophages (Abrashkin et al. 2006). Even for macrophages that remain within the ligament, it seems to matter what types of cell surface receptors they express. Sautter et al. (Sautter et al. 2006) showed that mice lacking chemokine receptor 2 (CCR2, a particular macrophage surface receptor) may sustain more noise-induced PTS than wild-type controls. This suggests there exists some type of communication between these cells and the organ of Corti. Alternatively, if the macrophages act solely to preserve the spiral ligament, the results are consistent with dependence of the organ of Corti on the condition of the ligament.

3.3 Hair Cells as Generators and Targets of ROS/RNS

3.3.1 Noise and Hair Cells

It is actually not obvious why excessive noise should impose ROS/RNS stress on cochlear hair cells. At least for sub-traumatic exposures, all hair cells have to do is passively gate transducer currents through their apical and lateral membranes. Even the outer hair cell (OHC) motility "amplifier" utilizes the regenerative simplicity of a molecule (prestin) that transitions between two conformations, without adenosine triphosphate (ATP) hydrolysis (Dallos et al. 2006). The limiting ability is likely to be maintenance of appropriate Ca^{++} levels. Calcium enters the cell through the transducer channel where it modulates the adaptation state of the hair bundle (Ceriani and Mammano 2012) and is removed by ATPase pumps, also within the stereocilia. These pumps may be overwhelmed during noise exposure. In active hair cells, intracellular Ca^{++} levels increase from multiple sources, including release from Hensen's bodies and influx through multiple membrane channels, including

ATP-gated Ca^{++} currents in the hair cell lateral membrane. The increased Ca^{++} drives glutamate release from inner hair cells (IHCs) and modulates somatic contractions of OHCs. Yet the allowable range for intracellular Ca^{++} is very narrow. For reasons that hardly seem adaptive, excess Ca^{++} in hair cells is taken up by mitochondria, where it can trigger increased and unbalanced superoxide and NO production (Shi et al. 2003; Shi and Nuttall 2003). The most straightforward action of Ca^{++} may be to alter mitochondrial membrane potential through accumulation of positive charges (Arundine and Tymianski 2004). Böttger and Schacht (Böttger and Schacht 2013) further suggest that a particularly compelling point of intersection between Ca^{++} and mitochondrial function is the Ca^{++}-modulated Krebs cycle enzyme α-ketoglutarate dehydrogenase. Compromised mitochondria may reach a threshold "permeability transition," at which point they release cytochrome C, apoptosis-inducing factor (AIF), and endonuclease G and engage various caspases, tipping the balance toward apoptosis (Han et al. 2006). Aside from its effects on mitochondria, cytoplasmic Ca^{++} engages proteases and phospholipases, with subsequent release of prooxidant arachidonic acid. The role of Ca^{++} in hair cell injury is supported by the benefits of both L- and T-type Ca^{++} channel blockers against noise- and age-related hearing loss in mice and perhaps humans (Shen et al. 2007; Uemaetomari et al. 2009).

A substantial literature highlights the value of cochlear pharmacotherapies targeted against both ROS and RNS (for reviews, see Le Prell et al. 2007; Ohlemiller 2008; Le Prell and Bao 2012), clearly implicating these in hair cell injury and supporting the feasibility of this approach (see also Chaps. 9, 12, and 16). The role of NO in hair cell injury is perhaps a bit ironic, in that one major role of nNOS (the major hair cell isoform) is taken to be negative feedback on Ca^{++} entry (Shen et al. 2003). NO may readily exceed adaptive levels, partly because iNOS is also activated (Yamamoto et al. 2009). Besides giving rise to peroxynitrite, it overactivates PARP1 (poly-ADP-ribose polymerase-1), a DNA repair enzyme that depletes cellular ATP and NAD^+ energy currency in the process (Schreiber et al. 2006).

How can the protective systems of cochlear cells become so easily unbalanced and maladaptive? The conditions we create in the laboratory, cochlear cellular stress responses simply did not evolve to resist. What we create in the lab—or perhaps in recreational and occupational settings—were never experienced by cochlear cells throughout evolution. Since we have no "head-to-head" comparison of the hardiness of human and animal hair cells, we should be prepared for fundamental differences in the investment different species may make in hair cell survival or repair. Indeed, a host of studies suggest intrinsic differences in vulnerability to noise. Susceptibility to noise-induced PTS varies across inbred strains of mice (e.g., Yoshida et al. 2000; Davis et al. 2001) and between rodent species. CBA/Ca mice and albino Sprague–Dawley rats appear more vulnerable than pigmented guinea pigs (Duan et al. 2008), which appear more vulnerable than humans (Liang 1992). Notably, this calculus takes into account only hearing thresholds: For a given amount of PTS, CBA/Ca mice actually lose fewer hair cells than do rats, guinea pigs, and perhaps humans (Ohlemiller 2012). The surviving cells are perhaps simply not repaired (Wang et al. 2002).

3.3.2 Ototoxicants and Hair Cells

The primary cochlear targets of aminoglycoside antibiotics are hair cells, particularly OHCs (Poirrier et al. 2010; Xie et al. 2011; Heinrich and Helling 2012). This does not appear to reflect greater uptake by hair cells, but rather is particular to the biology of hair cells versus non-sensory cells. Generation of ROS in hair cells following gentamicin has been demonstrated using fluorescent or other indicators (Clerici et al. 1996; Hirose et al. 1997; Jiang et al. 2005). Aminoglycosides can directly catalyze ROS formation, as well as activate iNOS, but also appear to cripple antioxidant protections. As in the case of noise, mitochondrial injury and NOX activity play a major role in ototoxicant-related oxidative stress, and generally similar cascades trigger apoptosis. By contrast with noise (Yamamoto et al. 2009), NFκB appears to play a more unambiguous role in endogenous protection. Iron chelators and a wide range of antioxidants have proven effective against aminoglycoside-induced hair cell loss (Le Prell et al. 2014).

Cellular targets and ototoxic mechanisms of the antineoplastic cisplatin overlap with those for aminoglycosides, except that cisplatin may more aggressively deplete cellular antioxidants and engage more NOX isoforms. It may also more readily promote hair cell Ca^{++} entry via transient receptor potential vanilloid-1 (TRPV1) channels (Mukherjea et al. 2008) (see also Chaps. 10 and 11).

3.3.3 Aging and Hair Cells

We are born with cochleas possessing an excess of strial and ligament pumping capacity and, to some degree, redundant afferent neurons. Regarding hair cells, if only IHCs and behavioral hearing thresholds are considered, we may be able to tolerate up to 80 % losses (Lobarinas et al. 2013). However, thresholds appear fairly intolerant to OHC losses (Hamernik et al. 1989). Mammalian cochlear hair cells die once and do not regenerate. Each day, they are confronted anew with the decision whether to die and must "decide" based on a balance of factors. While one factor just might be the host's taste in music, the bulk of the decision likely rests on the cells' overall mitochondrial status. That is, to what extent are "enough" mitochondria functional? Over the course of evolution, most mitochondrial genes have migrated to the nucleus. The remnant mitochondrial genome is small, fragile, poorly protected, and under-repaired (Böttger and Schacht 2013). Time imposes wear and tear from noise and ototoxicants, yet also from radiation and stray reactions, so that cumulative mitochondrial DNA damage is central to many notions of aging (Sohal and Orr 2012). What then follow are events similar to the sequelae of a noise exposure: a series of oxidative cascades and injury to lipids, proteins, and DNA. Human temporal bones reveal that presbycusic ears tend to be ears with more damage to mitochondrial DNA (Bai et al. 1997). Energetically disadvantaged cells do everything more poorly, including a poorer job of maintaining antioxidant defenses.

The most significant lost capacity of old postmitotic cells may be defined as lost "operating margin": reduced ability to respond to stress and a more precarious homeostatic balance. Extension of this operating margin is central to the touted advantages of caloric restriction and its mediators (Ohlemiller and Frisina 2008; Someya et al. 2010; Han and Someya 2013) (see Chaps. 13 and 16). Cochlear oxidative imbalance has been diagnosed in aging animals (Jiang et al. 2006), and antioxidant genes may be notably up- or downregulated with age in a manner consistent with chronic oxidative stress (Lautermann et al. 1997; Staecker et al. 2001; Tanaka et al. 2012). At present, evidence for the value of long-term pharmacotherapies against presbycusis is mixed (Poirrier et al. 2010; Heman-Ackah et al. 2010; Böttger and Schacht 2013).

3.4 Cochlear Afferent Synapses as Generators and Targets of ROS/RNS

3.4.1 Noise-Related Synaptopathy?

Until recently, cochlear noise injury was largely a hair cell story. Permanent threshold shifts were compared with hair cell loss, with general correspondence between the two. Transient swelling of the inner hair cell afferent synapses was a curious detail, perhaps with some role in temporary threshold shifts (TTS) (Pujol et al. 1991, 1993). Now, quite a stir has been raised over the discovery in mice that noise that induces a robust TTS can yield permanent synaptic injury that effectively deafferents IHCs and promotes progressive neuronal loss (Kujawa and Liberman 2006, 2009). These effects can occur independently of hair cell loss. The functional effects do not manifest in threshold shifts, but rather in suprathreshold decreases in evoked potential amplitudes. If the basic findings apply to humans, expected perceptual correlates might include degraded speech reception in noisy backgrounds. However, the extent of this pathology, termed "synaptopathy," varies by species, being less pronounced in guinea pigs and rats than in mice (Liu et al. 2012; Shi et al. 2013), and its applicability to humans remains speculative. One reason it may not readily translate is that the human cochlear nerve is anatomically distinct from the other species mentioned with regard to myelination and the presence of gap junctions between the somata (Tylstedt et al. 1997; Tylstedt and Rask-Andersen 2001; Glueckert et al. 2005). These features could increase sound-driven activity and promote the ultimate survival of human afferent neurons, *even if they have been disconnected from their hair cell targets*. Additional reasons for doubt derive from comparative neuronal counts in aged temporal bones of humans and mice (Makary et al. 2011; Sergeyenko et al. 2013). The similar trends by species—suggesting a linearly progressing loss of afferent neurons over the life-span in each—seem at odds with an expected skewing of human counts to lower values. The "absent" data points would represent the effects of undiagnosed noise exposure in the human sample. The verifiably unexposed mouse population would not display such an effect.

3.4.2 Synaptopathy and Aging

Even if not related to noise exposure, the notion of synaptopathy nevertheless fits with recognized features of aging temporal bones (Makary et al. 2011; Sergeyenko et al. 2013) and with aging trends in hearing. The single predominant complaint offered by the aged about their hearing is that environmental sounds (i.e., speech) are plenty loud, but the signal cannot be extracted from the noise (Frisina 2001, 2009) (see also Chap. 14). In the most severe cases, a diagnosis of neural presbycusis may be applied, but some degree of deficit may be nearly universal.

3.4.3 Mechanisms of Synaptopathy

What may drive the loss of afferent neurons whose target cells are still intact? The initial injury may involve excitotoxic effects of glutamate, which are oxidative and nitrosative in nature (Rego and Oliviera 2003; Gu et al. 2010; Kostandy 2012), and recapitulate the Ca^{++}-based injury processes described above for hair cells. All glutamate receptor subtypes operating at the IHC afferent synapse (AMPA/kainate, NMDA) can contribute to this process. Ironically, the excitotoxic cascade likely involves NO generated by nNOS that is "intended" to protect against excess Ca^{++}, yet which imparts net injury. Another surprising aspect of this story relates to NFκB. Following noise exposure, NFκB may contribute to broad injury of the organ of Corti and lateral wall through inflammation (Yamamoto et al. 2009) (for detailed discussion, see Chap. 19). At the IHC/afferent synapse, however, NFκB appears to serve as a brake on Ca^{++} entry. NFκB knockout mice show accelerated loss of afferent synapses fitting the pattern of synaptopathy (Lang et al. 2006). While synaptopathy may involve an initial stage that is excitotoxic, it may require additional processes that irreparably sever trophic signals that support dendritic connection and neuronal survival.

3.5 The Cochlear Lateral Wall as Generator and Target of ROS/RNS

3.5.1 Homeostasis of Endolymph and Perilymph Composition

The intracellular-like fluid that fills the scala media is termed endolymph, while the extracellular (and CSF-like) fluid that fills the scala tympani and vestibuli is termed perilymph. Neither endolymph nor perilymph appreciably "flow," nor are they "pumped" (Patuzzi 2011a, b). Instead, water passively adjusts to ion gradients that are established locally, largely by the action of the stria vascularis. Cells of the organ of Corti that insert into the reticular lamina are bathed in endolymph at their apical

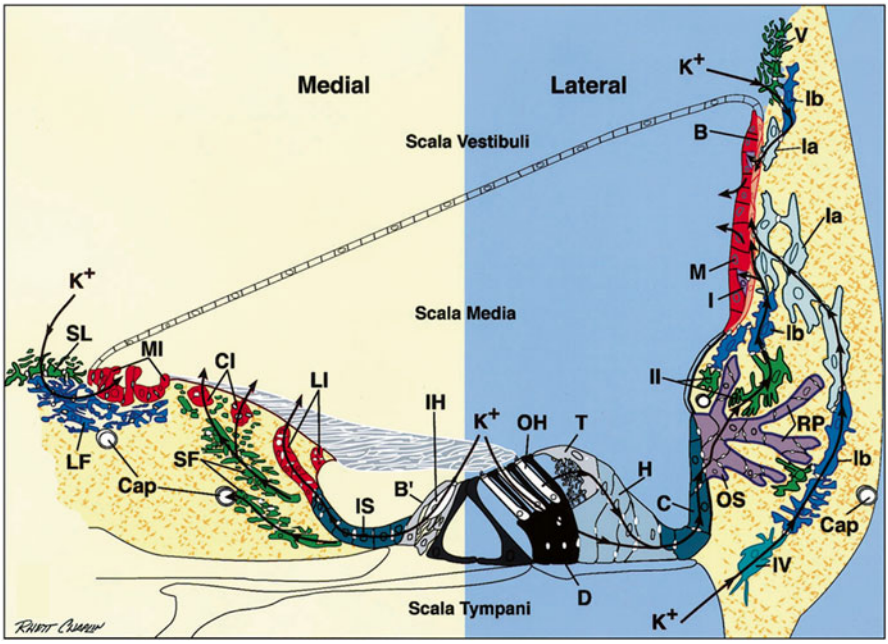

Fig. 3.2 Schematic radial view of the organ of Corti and adjacent lateral wall. *Arrows* indicate posited medial and lateral transcellular routes for K^+ effluxed from inner and outer hair cells (IH, OH) during auditory transduction. *B* strial basal cell, *B'* border cell, *Cap* capillary, *C* Claudius cell, *CI* central interdental cell, *D* Deiters cell, *H* Hensen cell, *I* strial intermediate cell, *IS* inner sulcus cell, *M* strial marginal cell, *MI* medial interdental cell, *LF* light fibrocyte, *LI* lateral interdental cell, *OS* outer sulcus cell, *RP* root process of outer sulcus cell, *SF* stellate fibrocyte, *SL* supralimbal fibrocyte, *T* tectal cell, *Ia*, *Ib*, *II*, *IV*, and *V* types of spiral ligament fibrocytes (From Spicer and Schulte 1998. Reprinted with permission Elsevier Publishing)

surface and by perilymph on all other surfaces (Fig. 3.2). All nutrients must therefore reach these cells via one of these fluids, and all waste must be carried away by one of these. Endolymph, while serving as the critical source of K^+, is generally not held to be the major source of nutrients nor the means by which metabolic waste is removed. Instead, perilymph must fill this need. Perilymph also serves as the milieu of the intercellular spaces of the spiral ligament and so is in free communication with the capillaries of the ligament. While tissues are typically composed such that most cells lie within 20 μm of a capillary, in the cochlea this distance may easily exceed 100 μm. This seeming problem, created perhaps by the need to separate mechanosensitive receptor cells from "noisy" capillaries, appears largely solved by a combination of factors. First is the evolutionary innovation of moving much of the work of pumping critical ions up their electrical and concentration gradients from hair cells to the lateral wall. This allows hair cells to operate at a lower metabolic rate than even non-sensory cells (Nakai and Hilding 1968). Another factor may be reliance on a network of gap junctions that connect nearly all cells lateral to the OHCs, through the inferior ligament, to the superior ligament, and finally to the

cells of the stria (Wangemann 2006; Zhao et al. 2006; Nickel and Forge 2008). This web of connected cells is discontinuous only within the inferior ligament where ions and macromolecules released by outer sulcus/root cells (the epithelial junctional network) must again be actively taken up by type II fibrocytes (the connective tissue junctional network). Yet the web extends to the stria itself, ultimately linking type I fibrocytes of the ligament to strial basal and intermediate cells. This arrangement promotes K^+ recycling to the stria yet also offers the possibility that both strial and ligament capillaries could support organ of Corti function. Indeed, work in connexin 30 (Cx30) mutant mice has revealed transfer of glucose from the stria to the ligament that is abolished in the mutants (Chang et al. 2008) (Fig. 3.5). Traffic though the gap junctional network must be two way, presumably with K^+ and metabolic waste diffusing away from the organ of Corti and oxygen and glucose diffusing toward the organ. This traffic may be gated to some extent by ATP, inositol triphosphate (IP_3), and Ca^{++} (Zhu and Zhao 2010). Both peroxide and NO reduce gap junctional conductance, so that injury from oxidative stress may be amplified through simultaneous effects on both the organ of Corti and lateral wall. It has long been clear that chronic dysfunction of lateral wall gap junctions promotes degeneration within the organ of Corti (Nickel and Forge 2008; Xu and Nicholson 2013). Mutations of connexin 26 (Cx26) account for roughly half of all cases of nonsyndromic hereditary deafness and are associated with such degeneration. The potential significance of transient disruption of gap junctions due to noise, ototoxins, or inflammation for permanent hearing loss is an emerging idea that may heighten interest in acute lateral wall injury (see below).

3.5.1.1 Limitations Posed by Vasculature

Distinctly different vascular loops serve Rosenthal's canal and spiral ganglion cells versus the lateral wall and organ of Corti (Axelsson 1988; Axelsson and Ryan 2001). Capillaries of the stria and spiral ligament also likely serve the metabolic needs of the organ of Corti. The primary route is probably through the ligament to the lateral organ or through the scala tympani to the organ of Corti. The nutritive needs of the medial organ of Corti (particularly IHCs) may also be met by capillaries in the spiral limbus. The vessel of the basilar membrane is not maintained in adulthood in most non-primates, and its appearance even in primates is highly variable; thus it is unlikely to serve as the primary source of blood flow to the organ of Corti.

It is widely supposed that the sheer distance of the blood supply from hair cells presents a problem under demanding conditions such as noise exposure. Moreover, noise exposure appears to reduce cochlear blood flow via both inflammatory and oxidative mechanisms (Miller et al. 2003; Nakashima et al. 2003; Arpornchayanon et al. 2013). Noise-related vasoconstriction does not seem to occur within the stria. Rather it may occur in the superior ligament, in the type V fibrocyte region (Dai et al. 2011) (Figs. 3.2 and 3.3) where vessels that feed the stria and ligament emerge from the boney cochlear turn boundaries. This may explain why variation in blood

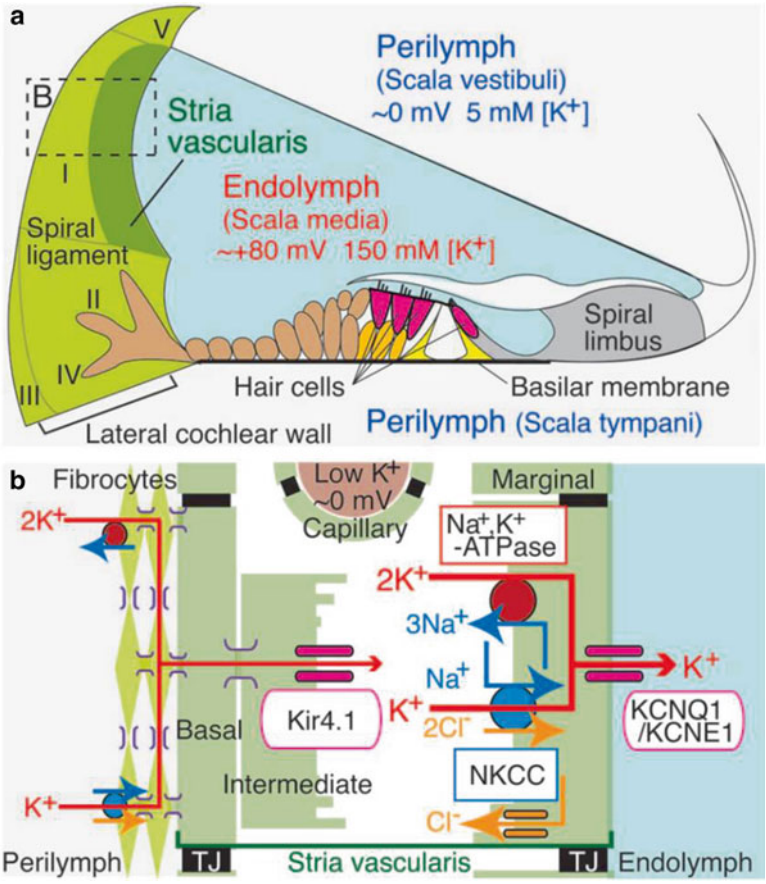

Fig. 3.3 Functional schematic structure of the cochlear duct with the lateral wall. (**a**) Appropriate ionic composition of the endolymph and the EP require an ion barrier lining scala media, the intrastrial space, and strial capillaries. A nearly continuous cellular network guides K^+ from the organ of Corti through finger-like root cells into (primarily) type II and I fibrocytes. Type II fibrocytes must take up K^+ from the extracellular space around root cells. Five major types of fibrocytes are indicated by roman numerals. (**b**) Enlargement of the boxed area in A depicts the cells and components needed to generate the EP. Type I fibrocytes, strial basal cells, and intermediate cells are joined by gap junctions composed mostly of connexins 26 and 30. K^+ enters the intrastrial space through Kir4.1, then through marginal cells via Na^+/K^+-ATPase, the NKCC ion exchanger, and KCNQ1/KCNE1 channel complexes. *TJ* tight junction (Reprinted with permission from Nin et al. 2008)

flow (velocity) can be detected in the stria, yet also how vasoactive agents (e.g., TNFα, 8-iso-prostaglandin $F_{2\alpha}$) that are likely generated *within the cochlea* can nevertheless impact strial blood flow. Some capacity for local regulation may also exist within the capillaries of the ligament (Wu et al. 2011). The precariousness of the vascular supply to the organ of Corti may represent the single overarching weakness that exposes the organ to injury (see also Chap. 8).

3.5.2 Role of the Stria Vascularis in Hearing

After K^+ has served its primary function of mediating hair cell sound responses, it is released into the fluid space around hair cells. Na^+/K^+ ATPases in both the type II fibrocytes of the spiral ligament and strial marginal cells create critical sinks for K^+ recycling (Hibino and Kurachi 2006; Hibino et al. 2010) (Fig. 3.3). The type II fibrocytes create the first sink by lowering extracellular K^+ levels at the root cell/type II interface. This yields a concentration gradient for K^+ that facilitates its movement from the organ of Corti, through the epithelial gap junctional network, to the root cells. Once the type II fibrocytes have taken up K^+, it is not actively *pumped* through the connective tissue gap junctional network, but rather moves down the concentration gradient created by the sink within the intrastrial space, created by strial marginal cells. The stria creates both the high, positive, endocochlear potential (EP) (+90–100 mV) and the high K^+ levels of the scala media. Cochlear neuronal response thresholds and spontaneous activity, in turn, depend on the EP (Patuzzi 2011b). As might be expected, factors that reduce the EP, such as aging, and events such as aminoglycoside exposure, noise exposure, and loop diuretics, elevate physiological thresholds with a dependence of 0.5–1.0 dB/mV (Sewell 1984; Schmiedt 1993; Ohlemiller 2009) (Fig. 3.6). With the exception of aging and noise exposure in some strains and ages of mice (Ohlemiller et al. 2011), EP reduction generally appears temporary.

3.5.2.1 Requirements for an Endocochlear Potential

The essential ingredients of EP generation revolve around the availability of K^+, the machinery to move K^+, and the tight compartment boundaries to keep K^+ corralled in the appropriate space.

Machinery for EP Generation

In the same way that an automotive fuel pump cannot work if the gas tank is empty, the machinery of EP generation must have K^+ to work with. There must be an intact connexin 26, 30, and 31 gap junctional network for K^+ to move from the organ of Corti, and type II fibrocytes must have functioning Na^+/K^+ ATPase to be able to take up K^+ in the inferior spiral ligament. In Cx26 knockout mice, the EP is abnormal and hair cells degenerate, yet the stria retains a normal appearance.

The strial machinery for EP generation is shown in Fig. 3.3 (Hibino and Kurachi 2006; Wangemann 2006; Nin et al. 2008; Hibino et al. 2010). K^+ is conveyed from spiral ligament into strial basal cells and intermediate cells by gap junctions. K^+ then exits the intermediate cells into the intrastrial space via KCNJ10 (Kir4.1) channels, driven by the steep gradient for K^+ that normally exists across this boundary.

It is this step—large K^+ flux across a high resistance—that produces the EP. This flux would not occur, however, if K^+ were not readily taken up into marginal cells via Na^+/K^+ ATPase and the $Na^+/K^+/Cl^-$ exchanger. K^+ is then released into the scala media via channels jointly formed from KCNQ1 and KCNE1. Note that as a consequence of the trilaminar construction of the stria, the generation of the EP and the establishment of high K^+ levels are accomplished in separate layers and actions. The situation in the cochlear scala media contrasts with that in the endolymph of the utricle, where a monolayer of marginal cell-like dark cells produces a high K^+ concentration but a very low EP.

Endolymphatic Boundaries

The boundaries of the scala media separate high-K^+ endolymph from low-K^+ perilymph and thus must be impermeable to uncontrolled ion flow. These boundaries are composed of all the cells that line the scala media, from the stria, to Reissner's membrane, to the luminal surface of the spiral limbus, to the reticular lamina, and then finally to the outer sulcus cells and spiral prominence (Figs. 3.2 and 3.3). Also, since the intrastrial space must maintain very low K^+ levels, tight junctions join marginal cells on the luminal side of the stria and basal cells on the abluminal side, so that this space is also ion-tight. Thus an additional set of boundaries surrounds the stria itself, setting off a fluid space that differs in composition from both endolymph and perilymph (Wangemann 2006). Finally, the endothelial cells that line strial capillaries are assumed to establish an ion-tight boundary that separates intrastrial fluid from blood plasma, since these also differ in ion composition (Wangemann 2006). The requirements of this boundary, and the implications of its failure, will be considered below.

3.5.2.2 How Constant Is the EP, and What Sets It?

If hearing thresholds depend on the EP, then should not the EP be highly constrained? How does the EP "know" what to be, and how constant is it for any normal individual? Regarding the former, no one has claimed to find a clear "reference" within the circuit that generates the EP. Regarding the latter, there are presently no data, since EP measurement is a terminal procedure conducted at a single point in time. We know that the EP varies among species (e.g., Conlee and Bennet 1993; Schmiedt 1993; Ohlemiller 2009) and also from cochlear base to apex, the EP being ~10 mV lower in the apex than in the base (Conlee and Bennet 1993; Ohlemiller et al. 2006, 2010). In guinea pigs, the spatial gradient correlates with endolymph Ca^{++} levels (Gill and Salt 1997), suggesting that Ca^{++} plays a role in EP regulation. For these things *not* to matter for thresholds, it might be necessary to offset EP reductions with compensatory increases in hair cell transducer conductance or in the gain of the cochlear amplifier. Modest reductions in the cochlear apex versus the

base may have little effect, based on evidence that the cochlear amplifier plays a smaller role in setting thresholds at low frequencies than at high frequencies (Sewell 1984).

Patuzzi (Patuzzi 2011b) suggests that some type of communication must match the "current source" that is the stria with the "current load" that is the organ of Corti. Known regulatory points for K^+ entry and exit from the scala media include the ATP-regulated KCNQ1/KCNE1 channel assemblies on the luminal surface of marginal cells and ATP-gated K^+ channels that line the reticular lamina and spiral prominence (Mockett et al. 1994; Lee et al. 2001). The stria releases ATP plus probably other signal molecules as well. Activation of ATP-gated channels has been shown to reduce the EP and lower the input impedance of the scala media (Housley et al. 2013). The recent data of Housley et al. indicate that activation of these channels (with acute lowering of the EP) may be one component of noise-induced TTS and that genetic impairment of this process may promote PTS. The TTS contribution may also reflect ATP-mediated changes in the micromechanics of the organ of Corti (Bobbin 2001; Bobbin and Salt 2005). The widespread paracrine effects of ATP release from many cell types are diverse and complex. No single comprehensive picture has emerged of exactly purpose is served by ATP and ATP-triggered Ca^{++} waves in the lateral organ of Corti. What is presently not supported by any evidence is any signal that passes from the organ of Corti to the stria. Thus, any communication between the stria and organ of Corti appears to be one way.

In the sections that follow, we summarize what can be inferred about lateral wall injury, largely based on animal models. In assessing the contribution of the lateral wall to PTS, we are faced with two problems. First, what do we measure? The standard for injury to the organ of Corti (or its remediation) is hair cell density. We know that OHCs are closely linked to hearing thresholds. Anyone practiced in cochlear morphometry can tell in a single glance at an appropriately stained surface preparation of the organ of Corti whether there is hair cell loss. But the lateral wall is far more irregular, and any two normally hearing individuals may differ greatly with respect to fibrocyte density, capillary density, or strial thickness. In inbred animal models such as mice, differences in these metrics due to insults can be detected, yet the relation of such losses to hearing thresholds may not be easily discernible. Instead, the metric of choice is the EP, which constitutes a useful "one-stop-shopping" metric for assessing the status of the lateral wall. For the EP to be normal, a host of ions must be properly regulated, and a large set of boundaries must be maintained. At least in inbred mice, changes in the EP correlate well with lateral wall morphometry (Ohlemiller and Gagnon 2007; Ohlemiller et al. 2011). But this still leaves a second problem: Even if the EP is restored after injury (as it typically seems to be), this may not mean that all critical support functions of the lateral wall have been restored. Even temporary EP reduction may herald processes that promote permanent damage to the organ of Corti during the period that the EP was reduced. If this is the case, it may ultimately be difficult to separate lateral wall injury from organ of Corti injury as a primary cause of hearing loss or as a locus of its remediation. The chemistries of injury of the lateral wall and organ of Corti

overlap extensively. Thus, finding the biochemical signature of oxidative or nitrosative stress in the lateral wall does not prove it is the critical site of injury. This is a frequent limitation of studies in this area (e.g., Hsu et al. 2000; Chen et al. 2008). Conditional knockout models for fibrocyte-specific proteins or of connexins restricted to the spiral ligament may help resolve these issues.

3.5.2.3 Noise and the Lateral Wall

While there is a moderate literature showing EP reduction by noise, few studies have dealt with EP recovery, and detailed parametric studies have been conducted only in mice (Hirose and Liberman 2003; Ohlemiller and Gagnon 2007; Ohlemiller et al. 2011). The most significant observation is that there is no *one* effect of a given exposure: Both acute and permanent EP changes depend on the exposure conditions, on the genetic background of the mice, and on their age. On some backgrounds (e.g., BALB/c), and in younger animals (<3 months), permanent EP reduction occurs more readily. Based on Fig. 3.4, the range of exposure levels that acutely reduce the EP may be strikingly broad, extending down to 92–95 dB SPL. Above 110–113 dB SPL, EPs appear uniformly low, typically below ~30 mV. In keeping with the observations of Hirose and Liberman (2003), this range may demarcate exposure levels that rupture the reticular lamina. This is a disruption the system almost certainly did not evolve to deal with, altering the shape of the organ of Corti and mixing endolymph and perilymph. When this occurs, loss of boundary integrity—not necessarily any injury or limitation of the lateral wall—will drive EP reduction. Moreover, the extent of PTS and pattern of cell loss will be determined by how the organ of Corti responds.

The data of Fig. 3.4 pose caveats for the design of experiments probing noise-induced PTS. Above and below the exposure intensity "rupture point," both acute injury and its recovery may entail quite different processes. The critical question for the experimenter is "What do you want to know?" It is perhaps reasonable to assault the cochlea with 120 dB SPL noise if the experimental question is *what can noise possibly do?* But if the goal is to model, say, typical human occupational noise, then using supra-rupture noise may yield misleading results. It should be emphasized that, for any given noise type, the noise level required to rupture the reticular lamina varies by species (Fredelius et al. 1987; Fredelius 1988; Henderson et al. 1993, 2008; Hirose and Liberman 2003). Thus one cannot pick a "rupture point" noise level for mice that was established in, say, guinea pigs. Finally, the notion of noise as a cause of intermixing of endolymph and perilymph is not strictly limited to rupture. A lingering—yet still critical—debate pertains to the creation of "holes" in the reticular lamina that may be left by technically sub-rupture noise exposures when OHCs die. Such holes have been found in chinchillas (Bohne and Rabbit 1983), even for fairly low-level exposures (Harding and Bohne 2004), but so far not in other species. Although holes are eventually covered by scars formed by neighboring supporting cells, OHC death may be amplified by toxic levels of K^+ while they persist.

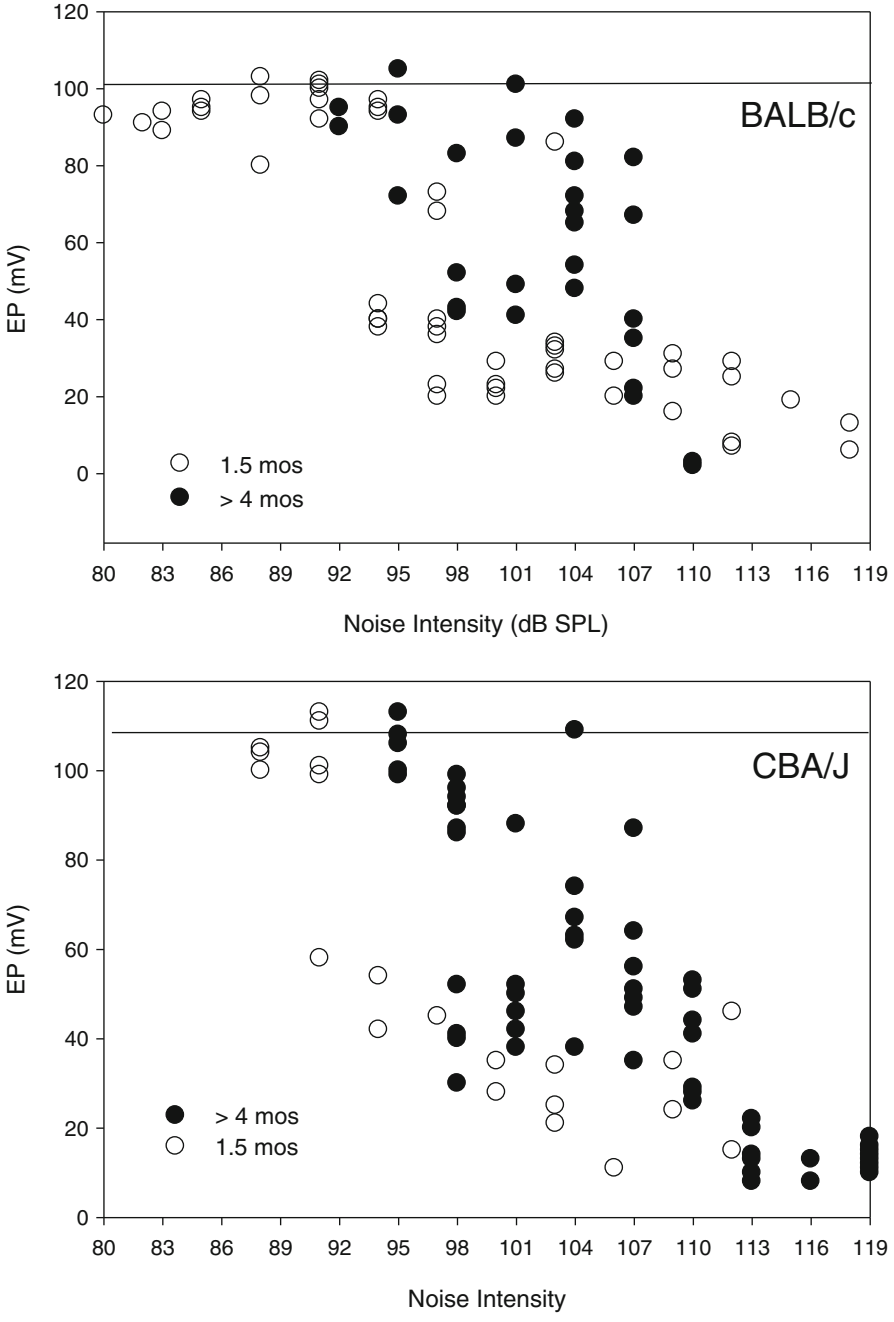

Fig. 3.4 Endocochlear potential is transiently reduced at surprisingly low noise levels. Basal turn endocochlear potential (EP) in young (1.5 months) or older (>4 months) BALB/c and CBA/J mice. *Horizontal lines* at tops of graphs indicate mean normal values for each strain. EPs were measured 1–3 h after 2 h of 8–16 kHz octave band noise at the intensities indicated. For each strain, the EP in younger mice was more vulnerable, but in each case, noise intensities causing acute EP reduction were surprisingly low (92–98 dB SPL). For both strains, exposures exceeding 110–113 dB SPL were associated with uniformly low EPs (<30 mV), likely indicating rupture of the reticular lamina

The cellular injury targets of noise in the lateral wall depend on exposure level but prominently include strial basal cells and most fibrocyte types in the spiral ligament (Ohlemiller and Gagnon 2007; Ohlemiller et al. 2011). More severe exposures cause clearer injury to strial marginal cells (Hirose and Liberman 2003). The common element of this injury is formation of intracellular vacuoles in strial basal cells and type II and V fibrocytes but shrinkage of type I fibrocytes. The most straightforward interpretation is that this indicates either intra- or extracellular buildup of some ion, most likely K^+, although a significant contribution of Na^+ cannot be ruled out. Noise may inhibit "downstream" (strial) K^+ transport and promote accumulation "upstream" in the inferior ligament. The pattern of vacuole formation indicates that some ion builds up *inside* of basal cells and type II and V fibrocytes but *outside* of type I fibrocytes.

How does this injury process begin? It is not a trivial question exactly *why* noise should cause pathology in the lateral wall. Both "inside-out" and "ligament-first" scenarios are possible. Inside-out refers to a pathologic cascade that begins in the stria and spreads to the ligament. This sequence has been suggested for aging gerbils (Spicer and Schulte 2002), where the pattern of cellular pathology is quite similar to that in noise-exposed mice. An inside-out sequence fits with the expectation—born out by observation (Spicer and Schulte 2005)—that strial marginal cell survival and function will be limiting for strial operation with age. By contrast, ligament-first refers to a scenario that begins in spiral ligament. Under this scenario, noise increases K^+ sinking by the organ of Corti, which leads to overload of pumping machinery for K^+ in the inferior spiral ligament. The excess K^+, failing to be taken up by the type II fibrocytes, then pools in the extracellular space of the ligament, potentially explaining shrinkage of type I fibrocytes. At some point, the amount of K^+ reaching the stria would be expected to decrease, and KCNJ10 K^+ current exiting strial intermediate cells may plummet. This might in turn interfere with the function of the $Na^+/K^+/Cl^-$ exchanger, leading to Na^+ and Cl^- buildup in the intrastrial space. If that is the case, vacuolation of strial basal cells could represent Na^+, not K^+, accumulation. For either scenario, widespread osmotic stress in the stria and the ligament probably acts as a driver of oxidative and nitrosative stress.

Mechanistic studies help place a molecular foundation under the events posited in the previous paragraph. Noise promotes ROS generation in both the stria (Yamane et al. 1995) and the ligament (Nagashima et al. 2010), so that there exists a clear oxidative stress component. Downstream events may include (1) redox-dependent phosphorylation of AMP-dependent kinases and c-Jun in the ligament (Nagashima et al. 2010, 2011); (2) upregulation of iNOS and NFκB (Shi et al. 2003; Yamamoto et al. 2009); (3) secretion of cytokines CCL2, IL-6, IL-1β, and TNFα by fibrocytes (Ichimiya et al. 2000; Fujioka et al. 2006); (4) release of cellular adhesion factors VCAM-1 and ICAM-1 (Yamamoto et al. 2009); and (**5**) leukocyte adherence to lateral wall endothelial cells, resulting in infiltration of the ligament by inflammatory cells (Shi and Nuttall 2007). Key effects on functional elements include reduced Na^+/K^+ ATPase activity in the stria and ligament (Morizane et al. 2005) and both reduced conductance and decreased expression of

connexins (Suzuki et al. 2009). Reductions in lateral wall Na^+/K^+ ATPase activity can be attenuated by antioxidants (Cheng et al. 2008). Effects of noise on Na^+/K^+ ATPase activity alone could represent the proximate cause of EP reduction. Nevertheless, a major question unresolved by these studies is "Why permanent hearing loss?" Given that the EP and Na^+/K^+ ATPase activity typically recovers (Nagashima et al. 2011; Ohlemiller et al. 2011), how might such a mix of pro-oxidative, pro-nitrosative, proinflammatory events drive the permanent organ of Corti injury required to explain noise-induced PTS? The most significant outcome of these events may be reduction of gap junctional conductances and downregulation of connexins. This may be caused by injury to fibrocytes from osmotic stress plus known antagonistic effects of peroxide, NO, and NFκB on gap junction conductance (Todt et al. 1999; Salameha et al. 2005). This effect also largely recovers, however, so how might temporary impairment of gap junctions promote permanent organ of Corti injury? Two primary mechanisms are supported by the literature, one invoking K^+ transport *away* from the organ of Corti toward the stria and the other invoking nutrient transfer away from the stria and ligament *toward* the organ of Corti. A tenet of the rather substantial literature on connexin-related genetic deafness is that loss of connexins promotes toxic accumulation of K^+ in the fluid spaces around hair cells (Minowa et al. 1999; Delprat et al. 2005). However, a more recently emphasized action of connexins is their role in conveying nutrients to the organ of Corti (Fig. 3.5, Chang et al. 2008). Above, we considered that the avascular organ of Corti is supplied nutrients (most notably oxygen and glucose) by perilymph, yet how efficiency may be gained if the gap junctional "highway" extending to the dense capillaries of stria vascularis also serves the organ. Gap junctions are permeable to a wide range of macromolecules up to ~1 kD (Matsunami et al. 2006; Xu and Nicholson 2013). Strial basal cells express glucose transporter 1 (Glut1) on their luminal membranes (Yoshihara et al. 1999; Ando et al. 2008), which seems ideally placed to transfer glucose from the intrastrial fluid to the connective tissue gap junctional network (Suzuki et al. 2009; Xu and Nicholson 2013). Other details that do not readily fit into this scheme are the unknown type and distribution of additional types of glucose transporters and the gap junctional "disconnect" that exists at the interface of root cells and type II fibrocytes (Wangemann and Schacht 1996; Wangemann 2002, 2006; Zhao et al. 2006). It is widely agreed that K^+ from the organ of Corti is normally taken up by transporters within type IIs. But how might glucose and other needed molecules be passed from the type IIs to the epithelial gap junctional network, and how do hair cells acquire them? Even though some details are lacking, evidence from mice (Fig. 3.5) and other considerations (Xu and Nicholson 2013) support the transfer of metabolites from the stria to the organ of Corti via gap junctions. If this transfer is interrupted by noise-related injury to the lateral wall for some critical period of time, permanent injury could result. By the time the EP recovers (24–48 h, based on our data), permanent damage may be done. Some of the therapeutic effects of antioxidants and NOS inhibitors against noise-induced PTS may derive from their ability to maintain Cx26 expression (Nagashima et al. 2010).

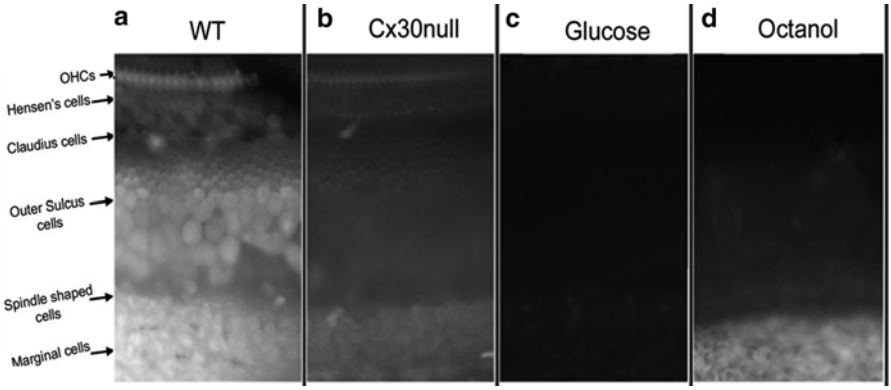

Fig. 3.5 Gap junctions are required for transport of nutrients from the stria and ligament to the organ of Corti. Images show dispersion pattern of a systemically injected fluorescent glucose analog, followed by excision of the lateral wall + organ of Corti and flattening for confocal viewing. Subjects were mice that were either WT for Cx30, Cx30 nulls, or WT mice treated with conventional glucose or the glucose analog following a Cx30 inhibitor (octanol). Approximate cell locations are indicated at left. Glucose transport from the intrastrial space to the organ of Corti was impaired in Cx30 nulls, and in WTs treated with octanol. Transfer of the glucose analog was competitively antagonized by larger dose of glucose (Adapted with permission from Chang et al. 2008)

If the foregoing arguments are correct, acute EP changes should predict the likelihood of noise-induced PTS, even if the EP recovers. To test this prediction, we identified in young BALB/c mice an exposure condition (8–16 kHz, 2 h, 95 dB SPL) that creates a mix of animals with noise-induced TTS or PTS. ABR thresholds and EPs measured 1–3 h after noise reveal a wide range of both acute threshold shifts (not shown) and EPs (Fig. 3.5) in these mice. At 1–3 h post-noise, we do not know which mice will ultimately show PTS. However, we have a large set of data from similarly exposed animals wherein ABR thresholds were obtained at both 1–3 h and 7 days post-noise. We are therefore able to guess, for similarly exposed animals, which acute threshold shifts measured at 1–3 h *predict* later PTS. Figure 3.6 shows a comparison of within-ear EPs and thresholds at the single frequency showing the greatest variance of thresholds (10 kHz). At this frequency, the largest 10 kHz threshold shift that recovers (based on archival data) is 37 dB SPL (thick dashed horizontal line). The four data points that lie well above this line correspond to EPs below 75 mV. Based on the archival data, these are probably PTS mice. Keeping in mind that the EP almost certainly would have recovered in these animals, we would assert that *there is no reason why acute EP and PTS probability should be correlated unless the acute EP reduction coincides with some other process that promotes PTS*. We propose that process is transient (24–48 h) disruption of gap junctions, either exposing hair cells to toxic K^+ levels, depriving them of glucose, or both. Since these data are indirect, purely correlative (noncausal) bases for the order in Fig. 3.6 cannot be ruled out.

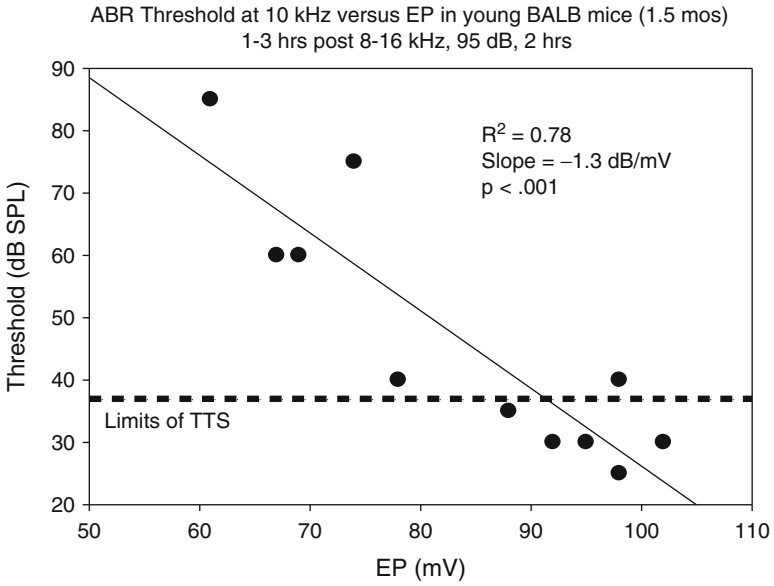

Fig. 3.6 Acute reversible endocochlear potential reduction following noise exposure predicts PTS. ABR threshold at 10 kHz versus EP in the same cochlea of young BALB/c mice 1–3 h after a 2-h noise exposure (8–16 kHz, 95 dB SPL). Under these conditions, acute thresholds and EPs are highly variable (see also Fig. 3.4). Our archival data suggest that under these exposure conditions the EP recovers, but there is considerable variability with respect to whether animals show only TTS or go on to develop PTS (as measured 7 days post-exposure). Because EP recording is a terminal procedure, we could not retest the same animals at 7 days. Instead, to infer which acute thresholds in the graph would likely have been associated with PTS, we compared ABR thresholds at 1–3 h and 7 days post-noise in archival data for young BALBs. Archived data suggested that acute thresholds above 37 dB SPL at 10 kHz (heavy *dashed horizontal line* in graph) are associated with PTS at multiple frequencies. Acute EP reductions and ABR thresholds were well correlated. Four animals showed thresholds above 60 dB SPL and EPs below 75 mV and likely would have later manifested PTS. Although indirect, these data support a link between *acute* EP changes and *permanent* threshold shifts

3.5.2.4 Aging and the Lateral Wall

Schuknecht defined strial presbycusis as hearing loss that results from strial degeneration and EP reduction (Schuknecht et al. 1974). This degeneration can arise independently of other age-associated degeneration, so that some presbycusis can be principally caused by strial pathology. In humans and animals, aging is reliably associated loss of strial cells, strial volume, and strial capillaries. Often this seems to have no functional consequence. The stria apparently begins with a great deal of excess capacity. Human and animal estimates indicate that roughly half can be lost before the EP declines (Schulte and Schmiedt 1992). Work in mice indicates that EP decline possesses both genetic and gender components (Ohlemiller 2009). Females seem to fare worse, particularly after menopause (Guimaraes et al. 2004; Ohlemiller et al. 2010). These findings match some claims for human strial

presbycusis (Gates et al. 1999; Hederstierna et al. 2007, 2010), as inferred from audiogram shape, since the EP cannot be recorded in humans. Another character suggested even by inbred (thus genetically identical) mice is a high level of variability, suggesting a prominent role for stochastic or environmental factors.

Based on work in gerbils (Schulte and Schmiedt 1992; Gratton et al. 1997), age-associated EP decline reflects loss of Na^+/K^+ ATPase activity in strial marginal cells. Yet this may largely result from general progressive impairment of these cells. Both human and animal observations indicate that the initial pathology of strial presbycusis manifests in the appearance and density of marginal cells (Schuknecht et al. 1974; Spicer and Schulte 2005; Ohlemiller 2009). The high level of metabolic activity of marginal cells makes them prime targets for oxidative stress. Since these cells apparently are not replaced, maintenance and repair (and perhaps redundancy) constitute the prime strategy for prolonging function. It follows that the robustness of antioxidant protections should impact the longevity of marginal cells. Presently, the best evidence for this is an increased tendency for EP decline in C57BL/6 J (B6) albino congenics (Ohlemiller et al. 2009). In normal B6 mice, marginal cells sequester melanin released from strial intermediate cells. A striking overall transfer of melanin from strial middle layers to marginal cells is seen when young and old B6 mice are compared. In the albinos, marginal cell loss with age is exacerbated, and the incidence of EP decline increases. Melanin is a multifunction protein, yet among its recognized actions are quenching of free radicals and chelation of metals that may promote oxidative stress.

3.5.3 Stria/Ligament Interactions

In aging animals, strial degeneration often coincides with degeneration of the adjacent ligament (Spicer and Schulte 2002, 2005). We have considered why this might be the case, in that strial dysfunction may promote ion imbalance both within and around the cells of the adjacent ligament. If type II fibrocytes remain intact and thus continue taking up K^+ at a high level, K^+ may continue to build up in the ligament even if the intrastrial "sink" for K^+ is eliminated. Old mice with measured EP decline show a thinner ligament than in controls (Ohlemiller 2009). What is still generally lacking is a sense of how disrupted are the critical functions of the ligament in such cases. While strial pathology seems to promote ligament pathology, there is little evidence to support the converse. That is, neither organ of Corti nor ligament degeneration seems to promote strial degeneration.

3.5.4 The Myth of Capillary Leak?

We said earlier that the walls of strial capillaries are expected to be impermeable to ions and macromolecules. This assertion logically follows the requirement for tight control of ions (especially K^+) in the intrastrial space. Strial capillary leak has been

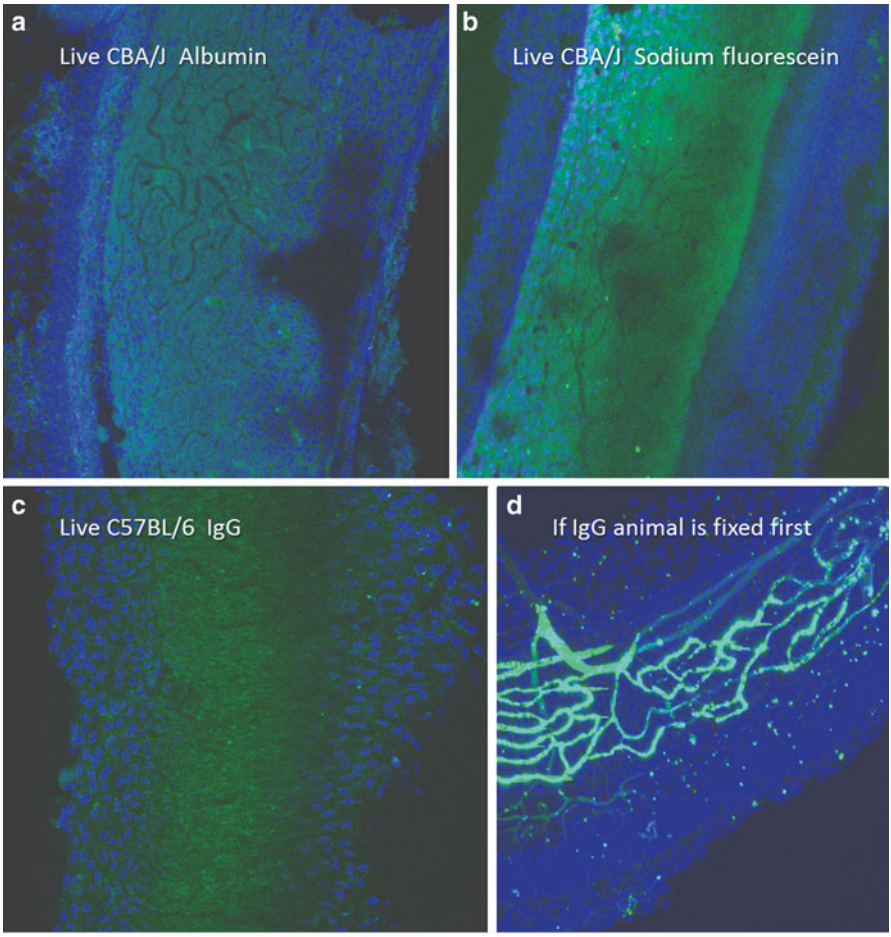

Fig. 3.7 Strial capillary "leak" may not be pathological. Dispersion of fluorescently labeled albumin (**a**), sodium fluorescein (**b**), or IgG (**c**) in the lateral wall of deeply anesthetized normal mice after direct injection of tracer into the left ventricle allowed to circulate for 5 min and then washed out with fixative. Dissected lateral walls were then viewed as whole mounts by confocal microscopy. In each case, the tracer was homogeneously distributed in the stria, but not in the ligament. Fixation of the cochlea by transcardial perfusion prior to injection of IgG (**d**) eliminated labeling except within the capillary lumen, suggesting that the apparent "leakage" of tracers was due to an active transport mechanism, taken to be transcytosis

"diagnosed" in animals using systemically injected horseradish peroxidase (HRP) (Sakagami et al. 1982) and fluorescently tagged albumin and IgG (e.g., Cohen-Salmon et al. 2007; Neng et al. 2013; Zhang et al. 2013). All these are seen to "leak out" into the intrastrial space under pathologic conditions. However, all can be observed in the intrastrial space under normal conditions as well (Sakagami et al. 1982; Suzuki et al. 2002) (Fig. 3.7). The most common mechanism does not appear to be uncontrolled paracellular leakage (i.e., between capillary endothelial cells), but active transport (transcytosis) across the endothelial cells (Dai and Steyger 2008).

Transcytosis appears to be very active in strial capillaries, more so than in ligament capillaries. It encompasses both highly regulated transport of receptor-bound molecules, as well as less stringent "fluid phase" transport. Given the prevalence of transcytosis, often missed by investigators, we know of no good evidence for unregulated leakage from strial capillaries.

Beyond the question of whether and how ions and macromolecules exit strial capillaries, there is the question of whether it would matter if unregulated paracellular leakage did occur. The ionic composition of intrastrial fluids and plasma differ mostly with respect to Na^+ and Cl^- (Wangemann 2006). Presently, there is little evidence that equilibration of ion gradients across capillary walls impacts the EP or the composition of endolymph. Water freely equilibrates across strial capillary walls, leading to strial swelling when there is an osmotic imbalance between plasma and the intrastrial fluids, such as when mannitol is applied systemically (Duvall et al. 1981; Santi et al. 1985). If strial marginal cells are the primary targets of noise and ototoxins, marginal cell dysfunction would cause K^+ to build up in the intrastrial space, which would then draw in water. Even a massively swollen stria largely regains a normal appearance (Ohlemiller and Gagnon 2007). The stria may be designed to allow for such volume changes. Tellingly, when mannitol causes strial swelling, the EP remains unchanged. In sum, even impressive changes in the appearance of the stria do not necessarily herald strial dysfunction.

3.6 Why Have Cochlear Oxidative and Nitrosative Stress Proven Resistant to Pharmacotherapies?

3.6.1 Which Targets?

The true scope of antioxidants and their reactions extends far beyond the typical reaction set shown in Fig. 3.1 (see also Chap. 4). If we wish to augment overall "antioxidant capacity," we might add one or more exogenous agents that mimic key antioxidants and thus amplify particular reactions. Yet it is not obvious which reaction may be most effectively amplified. Some branches may be more important than others, and some reactions (e.g., superoxide → peroxide) enzymatically convert one oxidant into another. Nevertheless, there may be key reactions to tweak. We noted that glutathione is maintained at high intracellular levels, suggesting that it possesses a wide therapeutic range and a monotonic dose–response curve. Glutathione acts both as a small molecule ROS scavenger and as a cofactor for antioxidant enzymes. Glutathione mimics have proven effective in remediating injury in a number of systems, notably including the inner ear (Le Prell et al. 2007) (see Chap. 8). Sohal and Orr (Sohal and Orr 2012) propose that the ratio of reduced glutathione to oxidized glutathione (GSH/GSSG) is a particularly critical metric for cell redox state, dwarfing other determinants. They further propose that bolstering the activity of peroxiredoxins, glutamate-cysteine ligase, and glucose-6-phosphate dehydrogenase (G6PD) is more likely to bear on GSH/GSSG ratios than many other enzymes.

3.6.2 Cellular Compartments

Distinct fluid compartments (either cellular organelles or isolated fluid compartments of the inner ear) carry out different ROS-generating reactions and may possess unique sets of antioxidants. These compartments may normally operate under different redox balances and thus may not respond equally favorably to a particular exogenous antioxidant. Beyond that consideration lie potential limitations regarding whether our chosen antioxidant can reach enter the desired compartment at sufficient concentrations, starting from the digestive tract. Liposome caging or binding of antioxidants to plasma carrier proteins, sugars, or particular hydrophilic moieties or emphasis on lipid-soluble antioxidants may help access-related issues.

3.6.3 Unbalancing of Innate Processes

A host of cell processes are modulated by redox-sensitive transcription factors. The net effect for any factor may be exquisitely redox-sensitive, because the target possesses multiple binding sites with opposite effects. As an example (Sohal and Orr 2012), we said that JNK is an important regulator of cellular responses to stress, participating in decisions of cells to repair or die (Veal et al. 2007; Bartosz 2009). JNK activity is oppositely affected by protein kinases ASK1 and MEKK1, which themselves have different sensitivities to oxidation. Whether the activation of JNK promotes cellular repair or apoptosis depends on whether one of both of ASK1 and MEKK1 are oxidized, so that the overall cell "decision" is based on the severity of oxidative stress. Yet JNK is just one signaling pathway among many, each possessing subtly different thresholds for opposing effects. Supposing each pathway constitutes one or more "votes" not merely for repair versus death but also for secretion, dedifferentiation, mitosis, migration, elongation, etc., exogenous antioxidants may disrupt this consensus. As Halliwell has emphasized (Halliwell 2009), the dosing dynamics for any single antioxidant will often be U-shaped, such that high doses are ineffective or even harmful. Partial success of antioxidant therapies very likely reflects disruption of the fine balance of multifaceted cell decisions *by cell type*. This is unavoidable. We should not be surprised by incomplete protection.

3.6.4 Different Requirements by Cell Type

We have seen that specific effector systems may have different redox thresholds for net benefit versus harm and may play different roles in different epithelia. One example is the very different effect of purinergic receptors in the lateral wall and lateral organ of Corti, where they appear protective, versus hair cells, where they may promote Ca^{++} overload. Another is the narrow net benefit-versus-harm balance

of NO in both the organ of Corti and lateral wall. We also noted wholly different effects of NFkB, serving to buffer Ca^{++} at the IHC afferent synapse versus promoting inflammation elsewhere. It will be difficult to tweak a given effector system at the correct levels at multiple locations.

3.7 Prospects for Improved Approaches to Preventing Hearing Loss

The best approach to acquired sensorineural hearing loss is not to lose cells in the first place. Pharmacotherapeutics taken by mouth—after exposure if possible—will be more workable than invasive and expensive gene therapies or siRNAs for this purpose. A host of compounds (steroids, anti-inflammatories, antioxidants, Ca^{++} antagonists, NOS inhibitors, antiapoptotics, purinergic agonists, Mg^{++}, trophic factors) and treatments (hypoxia, heat shock, sound conditioning, ototoxic conditioning) have been proven partially protective against acute cochlear insults in animals (Le Prell et al. 2007; Ohlemiller 2008; Le Prell and Bao 2012), some of these in small-scale clinical tests (see Chaps. 8 and 11). Across sites of injury (hair cells, synapses, lateral wall), Ca^{++} and nitric oxide represent a recurring theme. Thus, cocktails of agents targeting Ca^{++} and NO plus an antioxidant may merit special attention. Upstream factors identified in preconditioning research are also promising for pharmacologic mimicry because they may preserve the critical balance of downstream mediators of protection. As we noted, some "master switches" for expression of antioxidant genes have been identified, including Nrf-2 and Hif1α. These also constitute promising points for intervention, as they may also preserve the balance of downstream factors. While steroids appear beneficial, it is not clear by what pathway(s) they operate (Trune 2010), and they tell us little about whether cochlear invasion by macrophages is harmful or beneficial. Some of the signaling pathways we might engage vary so widely by cell type and seem so finely tuned that the best we can hope for is compromise. With regard to countering genes that promote acquired hearing loss, genome-wide association studies suggest that presbycusis and noise-induced PTS risk probably accumulate from multiple genes with small effect sizes. Rather than attempting to counter individual gene effects, compounds that target downstream injury cascades common to many "pro-PTS" or "pro-presbycusis" genes may render the broadest benefits.

It is doubtful that component ratios or precise formulations optimal for humans can be determined in animals, although animals are irreplaceable for proof of concept. Preventing slow, cumulative changes in hearing through pharmacologic approaches faces likely barriers of patient motivation and undesirable side effects. Hearing loss is more abstract, and perhaps more readily marginalized by patients, than is hypertension, diabetes, or even vision loss. The idea of preventing *future* hearing loss—whether from noise or aging—by any medication regimen may face a greater subjective barrier of skepticism than medications targeted at other health risks.

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Chapter 4
Antioxidants and Their Effect on Stress-Induced Pathology in the Inner Ear

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Abbreviations

ALCAR	Acetyl-L-carnitine
ALS	Amyotrophic lateral sclerosis
AR	Adenosine receptor(s)
ARHL	Age-related hearing loss
BDNF	Brain-derived neurotrophic factor
CDDP	Cisplatin
COX-2	Cyclooxygenase-2
Cyt c	Cytochrome c
DFO	Deferoxamine
DHB	Dihydroxybenzoate
eNOS	Endothelial nitric oxide synthase
ETC	Electron transport chain
FDA	Food and Drug Administration (US Federal Agency)
GDNF	Glial cell-derived neurotrophic factor
GSH	Glutathione
GTPase	Guanosine triphosphate hydrolase
HNE	4-Hydroxyneonenal
H_2O_2	Hydrogen peroxide

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OH^-	Hydroxyl ion
ICAM-1	Intercellular adhesion molecule-1
IHC	Inner hair cell(s)
iNOS	Inducible nitric oxide synthase
JNK	c-Jun n-terminal kinase
MET	Methionine
mtDNA	Mitochondrial DNA
NAC	*N*-Acetylcysteine
NADPH	Nicotinamide adenine dinucleotide phosphate
NIHL	Noise-induced hearing loss
NMDA	*N*-methyl-D-aspartic acid or *N*-methyl-D-aspartate
OHC	Outer hair cell(s)
PTS	Permanent threshold shift
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SOD	Superoxide dismutase
Superoxide	O_2^-
TTS	Temporary threshold shift
XO	Xanthine oxidase

4.1 Introduction

The evolution of aerobic metabolism led to the production of reactive oxygen species (ROS) in biological tissue. This consequence has been observed in both plants and animals (Apel and Hirt 2004). Typically, ROS have the ability to cause damage via oxidation of proteins, DNA, and lipids. Because of this phenomenon, living systems have co-evolved a complex array of enzymatic and nonenzymatic detoxification (or scavenging) systems designed to counter the impact of ROS.

The observation that an inverse correlation exists between metabolic rate and life span is not new. It has been known for the better part of the last 100 years of empiric inquiry that higher metabolic rate in animals typically suggests relatively short life spans. This early finding helped formulate the "rate-of-living hypothesis" (Finkel and Holbrook 2000). However, it was not until the 1950s when Harman developed this idea by positing the "free radical theory of aging" (Harman 1956). Harman theorized that the senescent cell progressively becomes less able to mitigate the effects of intrinsically generated ROS that cumulatively damage cellular processes, including DNA damage and cell death (Harman 1956). Concerted investigation over the next decade revealed the ubiquitous presence of the enzyme superoxide dismutase (SOD) in cells whose only recognizable function appeared to be the scavenging of the superoxide anions, thereby lending credence to Harman's original hypothesis. Today, the "free radical theory" and the "rate-of-living hypothesis" have been subsumed into a common theoretical framework, which visualizes a variety of ROS sources (endogenous such as from mitochondrial function, exogenous such as from the impact of ionizing radiation). These highly reactive molecules form a yin-and-yang

relationship with antioxidant defenses (such as enzymatic systems like SOD and nonenzymatic mechanisms like glutathione). If maintained homeostatically, ROS are known to be involved in critical processes like cell growth and metabolism. However, when this balance is perturbed, impairments accrue which include a diminished proliferative response, defective host defenses, random cellular damage, and disturbed signaling systems and, if unchecked, ultimately undesirable consequences like age-related dysfunction and pathological outcomes like cell death and disease.

4.2 The Biochemistry of ROS

ROS are highly reactive molecules that are mainly derived from the function of the mitochondrial electron transport chain (ETC) (Alfadda and Sallam 2012). There is a near ubiquitous generation of superoxide anion from molecular oxygen by the ETC in all tissues and cells. Free radicals are generated in cells in various ways. These include (1) reduction–oxidation reactions involved in cellular metabolism. Here oxygen is reduced to hydrogen by electron transfer. As a result, small amounts of partially reduced intermediates are generated on which different numbers of electrons have been transferred from oxygen. These intermediates include hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\bullet -}$), and hydroxyl ion ($^{\bullet}OH$). Furthermore, they are generated (2) as a consequence of absorbing radiant energy and (3) during inflammatory reactions where these species are formed in short burst in leukocytes by a multiprotein complex in the cell membrane that utilizes NADPH oxidase for the redox reaction. In addition, (4) metabolism of drugs or other exogenous compounds can also yield free radicals such as what occurs when CCl_4 (carbon tetrachloride) is broken down to CCl_3. Finally, (5) transition metals like iron and copper can become electron acceptors to generate free radicals via the Fenton reaction (where ferrous iron is oxidized by hydrogen peroxide to ferric iron, a hydroxyl radical, and a hydroxyl anion. Iron is then reduced back to iron, a superoxide radical, and a proton by the same hydrogen peroxide). (6) Nitric oxide (NO) (an important signaling molecule in endothelium, in macrophages, and in neurons) can act as a reactive radical and can also be converted itself to highly reactive species such as peroxynitrite ($ONOO^-$), NO_2, and NO_3^- (Droge 2002; Kumar et al. 2010).

With particular reference to the inner ear, free radicals are thought to be responsible for ischemia–reperfusion injury and cellular aging. These reactive molecules also initiate autocatalytic reactions in which their target molecules are themselves converted into free radicals, thereby compounding cellular injury (Kumar et al. 2010). Typically, cells are capable of maintaining a nominal balance between the generation of free radicals and their detoxification and clearance by intrinsic antioxidant defenses. *Oxidative stress* is the term employed to denote a state where this balance is lost and free radicals accumulate without check and damage cellular organelles and their membranes (Kumar et al. 2010). The biochemistry of ROS is reviewed in detail in Chap. 2 by Leeuwenburgh. In the following sections, findings that indicate that ROS have significant physiological roles in cell function and are not exclusively pathological entities, are discussed.

4.2.1 Physiological Overview of ROS

Mitochondrial-derived ROS, particularly superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2), have been shown to regulate vascular diameter (Liu et al. 2003). In recent years, even more compelling evidence has come to light that implicates $O_2^{\bullet-}$ and H_2O_2 in vasoregulation and dysfunction. For example, Xue et al. reported recently that when the novel eNOS transcription enhancer AVE3085 was used in a porcine coronary artery model, the deleterious effect of asymmetric dimethylarginine (ADMA) was attenuated via increased NO synthesis (by eNOS activation) and decreased oxidative stress (by inhibition of $O_2^{\bullet-}$) (Xue et al. 2012). When Roque et al. investigated the effects of aerobic exercise on vascular changes in mesenteric and coronary circulation in hypertensive rats, they found that $O_2^{\bullet-}$ was intimately involved in NO signaling in these vascular beds (Roque et al. 2013). In pig retinal arterioles, scavenging of superoxide has also been demonstrated by Omae and colleagues to contribute in mitigating detrimental effects of homocysteine and bradykinin-induced vasodilation (Omae et al. 2013). With respect to H_2O_2, several studies in recent years have cemented its role in modulating vascular tone and diameter as well. For example, Ruh et al. reported recently that when the H_2O_2 scavenger was used in the rat intestinal circulation, villous perfusion was dramatically reduced. These were shown to coincide with changes in the diameter of the main arteriole supplying this vascular bed. In fact, the scavenger was shown to attenuate the hyperemia associated with induced intestinal inflammation (Ruh et al. 2000). In an elegant set of experiments, Chaplin and Amberg demonstrated that the vasoconstrictor angiotensin II (Ang II) was able to generate ROS via NADPH oxidase (Chaplin and Amberg 2012). Interestingly, in these studies, H_2O_2 was the primary ROS generated and promoted co-localized Ca^{2+} influx via protein kinase C (PKC)-dependent L-type Ca^{2+} activation (Chaplin and Amberg 2012). Furthermore, Thengchaisri and Kuo have also described how H_2O_2 administration at micromolar concentrations can cause coronary artery dilation via an endothelium-dependent mechanism involving cyclooxygenase-1-mediated PGE_2 release (Thengchaisri and Kuo 2003). In other experiments expounding on the underlying mechanism of H_2O_2-mediated vasculoactive phenomena, a cogent argument has also been made that the vasodilatory role of H_2O_2 is likely beneficial in the context of eNOS uncoupling that underlies impaired NO signaling in many vasculopathies (Kuo and Hein 2013). In the cochlea as well, a tantalizing link between ROS and vascular homeostasis has been hypothesized backed by evidence reported in recent years. For example, markers of ROS generation in the cochlea are known to be abundantly expressed when the organ is exposed to noise in parallel with noise-induced vasoconstriction (Talaska and Schacht 2007). Brown and colleagues have reported that antioxidants can prevent the impact of noise on cochlear blood flow (CBF) (Miller et al. 2003). These and other investigators have suggested that vasoconstriction in the cochlea may be a consequence of ROS generation as well as a trigger for it when it can further amplify free radical-induced cochlear damage by the phenomenon of "reperfusion injury" (Brown et al. 1989; Thorne and Nuttall 1989; Talaska and Schacht 2007).

Oxygen sensing is another critical need for a variety of cell types. In this instance, the ETC acts to respond to O_2 concentrations and releases ROS in response to hypoxia (Guzy and Schumacker 2006). In turn, the ROS act as signaling molecules to trigger various compensatory effects such as increased synthesis and stabilization of hypoxia inducible factor 1 (HIF-1). HIF-1 is the primary trigger to induce angiogenesis and augmentation of oxygen delivery to hypoxic tissues or cells. In the mouse cochlea, noise-induced PTS was reported to cause accumulation of HIF-1α by Chung and colleagues several years ago (Chung et al. 2004). Arnold and Lamm have also shown similar changes in the guinea pig inner ear where noise exposure caused steadily worsening hypoxia and hearing loss that resolved upon termination of the noise insult (Lamm and Arnold 1996).

In the immune system, ROS are critically involved in both innate and acquired immune defense responses. When the immune system is exposed to pathogens, robust ROS synthesis occurs as part of the "respiratory burst" in phagocytes that is believed to account for the death of the invading pathogen. Subsequent to this "rapid" response, ROS are also found to play a role in mediating the T-lymphocyte response. In these cells, ROS upregulate the intracellular signal transduction and decrease their activation threshold (Droge 2002).

Oxidative regulation in skeletal muscle tissue is critical because of the high energy needs to ensure efficient and effective contraction. Therefore, skeletal muscles generate especially large amounts of ROS derived from mitochondria (Alfadda and Sallam 2012). In this system, ROS generation is induced by contraction, insulin, and hypoxia. Studies indicate that ROS act as signaling intermediates during contraction (McConel 2012). There is also some empirical evidence suggesting that muscle activity has an impact on antioxidant defenses. Investigators have reported that acute exercise augments circulating levels of antioxidant enzymes (Hatao et al. 2006; Berzosa et al. 2011).

Recent investigations have underlined a previously less recognized area of ROS physiology. Studies have shown that ROS are also involved in regulating transcription and genomic stability. For example, the redox state has been implicated in the posttranslational enzymatic covalent modification of histone and nonhistone proteins via acetylation and deacetylation reactions (Rajendran et al. 2011). Furthermore, cell cycle regulation, responses to DNA damage, apoptotic signaling, and autophagy, all of which are intimately linked to genetic regulation, are also partly controlled by ROS status (Rajendran et al. 2011).

4.3 Overview of Human Pathology and ROS

The role of ROS as causes of or as part of the causal chain in human disease is vast and has been under extensive study, extending from almost the entirety of the previous century into this one. Traditionally, the term ROS is thought to refer to molecules that include superoxide, hydrogen peroxide, singlet oxygen, ozone, hypohalous acids, and organic peroxides (Nathan and Ding 2010). However, these are not the

only class of highly reactive molecules, and a large volume of data has now established that other classes of such species exist in the cell and cellular systems. These include reactive nitrogen species (RNS) (e.g., nitric oxide $NO^{\bullet}$ and NO_2), hydrogen sulfide (H_2S and its anion HS^-), and carbon monoxide (CO) (Halliwell 2006, 2009).

As mentioned, ROS arise from a variety of subcellular locations or sources (the mitochondrial ETC being one of these). Further, they are known to act as physiological signals or intermediaries in cellular processes that are homeostatic. The term "oxidative stress" has been used by many authorities to denote a state where the generation of ROS exceeds the needs of the cell or when the cell's ability to neutralize their reactiveness has been compromised (Halliwell 2006, 2009; Darrat et al. 2007).

Broadly, ROS are thought to represent a key factor of disease causality, in humans and animals alike. While it is beyond the scope of this chapter to review all the areas of human pathology where ROS are known to play a role, it is illustrative to briefly acknowledge their involvement in certain specific pathological states. These include malignant disease, cardiovascular disease, and neurological disorders (Brieger et al. 2012). The DNA damage associated with ROS reactivity is of particular note in the genesis of cancer where these molecules cause mutations in the DNA by base modifications, rearranging DNA sequences, miscoding DNA, inducing gene duplications, and activating oncogenes (Waris and Ahsan 2006). In addition, ROS have been implicated in carcinogenesis via cellular dysregulation, e.g., in renal cell carcinoma where ROS anomalously upregulate hypoxia response genes (Liao et al. 2007).

ROS have also been implicated in the etiology of a number of cardiovascular disorders. For example, vascular endothelial cells express several isoforms of the NAD(P)H oxidase called NOX. Increased expression of specific isoforms is a phenomenon noted in cardiovascular pathology. These oxidases are sources of ROS themselves. In ischemia–reperfusion injury, a well-known mechanism in cardiac pathology, NOX trigger the disturbed flow-mediated vasodilation that is a hallmark of this pathology. In hypertension, superoxide can lead to a reduced bioavailability of the potent vasodilator, NO. In addition, ROS are also involved in the proliferation and hypertrophy of vascular smooth muscle cells which leads to increased vascular resistance (Brieger et al. 2012).

In the nervous system, ROS are generated by microglia, astrocytes, and neurons. In low concentrations, these molecules are required for normal functioning of these cell types. In higher concentrations, however, ROS lead to neurotoxicity (Block et al. 2007; Sorce and Krause 2009). In Alzheimer-type dementia, amyloid metabolism can also lead to increased oxidant stress which is thought to amplify neuronal dysfunction death. In Parkinson's disease, NOX-derived oxidant stress contributes to degeneration of dopaminergic neurons (Block et al. 2007; Sorce and Krause 2009). Furthermore, ROS signaling has also been implicated in the causality of amyotrophic lateral sclerosis (ALS) with mutations in the superoxide dismutase 1 (SOD1; a key superoxide radical scavenger) being associated with familial form of ALS (Brieger et al. 2012). In the sensory nervous system, ROS have been shown to affect the retina and the lens in the eye leading to degenerative pathology in both structures (Brieger et al. 2012). Interestingly, heightened NOX_3 activity has been reported in hearing loss, in particular that associated with cisplatin ototoxicity (Banfi et al. 2004).

4.4 Otologic Pathology Pertinent to Oxidative Stress

The cochlea is especially sensitive to oxidative stress (Kopke et al. 1999), largely due to high metabolic activity. In the cochlea, the stria vascularis, the inner hair cells (IHCs), and the outer hair cells (OHCs), all have robust antioxidant defenses. In this context, it is important to note that the basal region of the cochlea is relatively more bioenergetic than apical regions. This partly explains why the basal turns of the organ of Corti incur more oxidant damage from injury or toxins than other areas (Thalmann et al. 1973). These ideas are schematically summarized in the accompanying figure (Fig. 4.1).

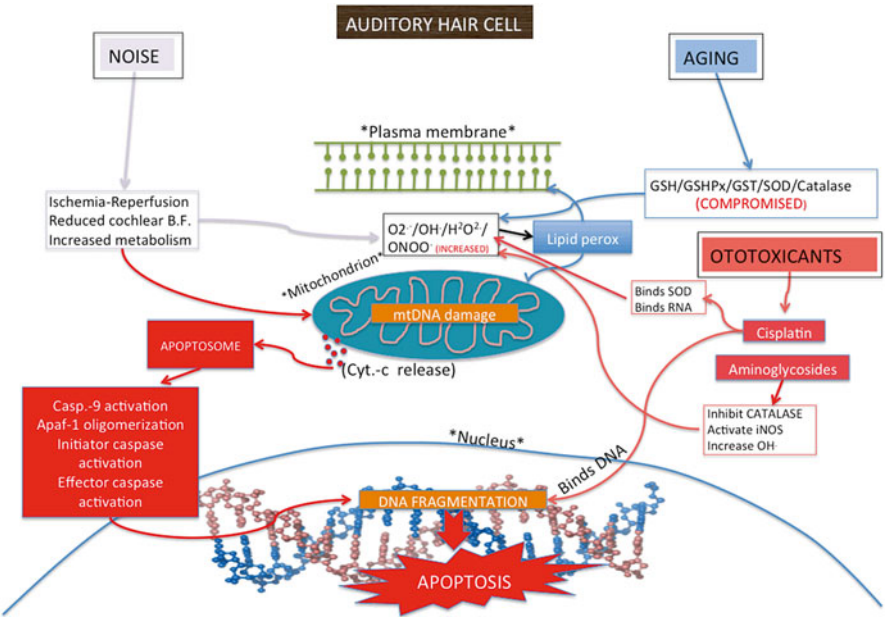

Fig. 4.1 A highly stylized auditory hair cell is drawn in this schematic. The cell is depicted to be under the stress of typical pathological triggers of noise, aging, and ototoxicants. Noise induces increased generation of free radicals via reduced cochlear blood flow, ischemia–reperfusion mechanisms, and increased metabolism also leading to increased free radical production by the mitochondrial electron transport chain. Aging, by reducing the effectiveness of antioxidant defenses (such as glutathione [GSH], glutathione peroxidase [GSHPx], glutathione *S*-transferase [GST], superoxide dismutase [SOD], and catalase), exacerbates oxidative stress. Ototoxicants like the aminoglycoside antibiotics or the cytotoxic compound, Cisplatin, act to interfere with antioxidant defenses, bind RNA and DNA, and activate iNOS, thereby stimulating the production of NO. ROS, in turn, damage the cell's plasma membrane as well as the membranes of organelles like the mitochondrion, including its genome. This outcome initiates the release of cytochrome c (Cyt c) and the caspase cascade leading to DNA fragmentation and apoptosis

4.4.1 Early Evidence

Historically, Hawkins and his colleagues were the first to note the parallels between cochlear damage observed in noise-induced injury and the type of otopathology seen in aminoglycoside toxicity (Hawkins 1973). They also reported that there was an apparent progression of the severity of injury progressing from cochlear base to its apex and from the OHC rows inward from the periphery. Also in the early part of the decade of the 1970s, Ylikoski reported swelling and distortion of the OHCs in guinea pigs exposed to aminoglycosides (Ylikoski et al. 1974). In addition, these investigators also reported significant subcellular distortions and damage in the OHCs. A few years later, Fredelius et al. examined the consequences on the cochlea of intense continuous noise exposure and reported that the stereociliary bundle was disrupted in IHCs and OHCs along with cuticular plate protrusions (Fredelius et al. 1988). In addition, these investigators described how the afferent nerve endings below the IHCs were swollen and translucent and that intracellular organelles (including mitochondria) were seen to degenerate. It is now clear that oxidative stress contributes to all of the above patterns of damage, and multiple recent reviews are available (Kidd and Linden 1975; Rybak and Whitworth 2005; Abi-Hachem et al. 2010; Poirrier et al. 2010).

4.4.2 Unique Vulnerability of the Cochlea to Oxidative Injury

As noted, the cochlea and organ of Corti are a highly energetic system. In order for the cochlea to transduce sound impulses, the stria vascularis is compelled to maintain an electrochemical gradient between the hair cells and the endolymph (Kopke et al. 1999). In addition, the OHC tuning of the basilar membrane is an energy-dependent process (Patuzzi 1996). Accordingly, the stria and the OHCs are mitochondrion-rich structures. Under physiologic conditions, the reactive species that are generated by the metabolic activity in these regions are effectively scavenged by endogenous mechanisms or oxidant neutralization (Thalmann et al. 1973). It is now understood that when conditions change for the worse, such as under the stress of intense noise exposure or the presence of ototoxins, there exists the potential that theses defenses will be overwhelmed resulting in oxidant-induced injury.

4.4.3 Noise and Reactive Species

It has been shown that noise exposure increases the rate of metabolism in the cochlea. If homeostatic systems are undisturbed or uncompromised, ordinary levels of noise exposure and its molecular effects are readily contained by the cochlear antioxidant defenses. Early studies demonstrated that antioxidant compounds and ROS scavengers could protect against noise-induced hearing loss (NIHL) (Quirk et al. 1994). However, overstimulation by intense sound can easily perturb the protective systems leading to increased glucose uptake and increased perilymphatic oxygenation

(Kopke et al. 1999). With further increases in incident noise, decreased CBF and declining endolymph oxygenation and glucose uptake are subsequently noted (Wangemann and Schacht 1996). This is fertile territory for a robust generation of ROS. Interestingly, NO has also been shown to operate as a neuromodulator in the central nervous system but can prove to be, in conducive settings, neurotoxicity, and indeed if enough of this short-lived molecule is generated (as outlined briefly above), it can act as an oxidant itself (Fessenden et al. 1994). In addition, calcium which is a tightly regulated messenger in the cochlea (as in many other tissues) can itself trigger ROS production when intracellular concentrations ($[Ca^{2+}]_i$) increase (Orrenius et al. 1992). The effects of noise on the inner ear are reviewed in detail by Altschuler in Chap. 7, and the genetics of NIHL are reviewed by Yamashita in Chap. 8.

4.4.4 Oxidant Stress and Cochlear Apoptosis

A large body of evidence suggests that oxidative stress in the cochlea, regardless of origin or trigger, will initiate apoptotic cascades in the hair cells as well as in the neurons of the spiral ganglion (Huang et al. 2000). As described, ROS-induced stress provokes the mounting of cellular defenses in the cochlea. Scavengers that are utilized, normally with good effect by the hair cells and neurons, include SOD, catalase, glutathione peroxidase (GPx), and glutathione (GSH). GSH, for example, acts to attenuate the oxidation of proteins by ROS. It is also known to function as a detoxifier of 4-hydroxynonenal (HNE, a plasma membrane lipid peroxidation byproduct that is very toxic). If HNE detoxification is reduced, increasing levels of this molecule in the cell can injure the mitochondrial membrane resulting in cytochrome c (Cyt C) release as has been described by classical experiments in recent years (Ji et al. 2001; Vieira et al. 2001; Rybak and Whitworth 2005) . Release of Cyt C is a well-understood trigger for apoptotic signaling to be initiated (Kruman et al. 1997). In other models of cochlear injury such as neurotrophin withdrawal, cisplatin exposure, hypoxia, or ischemia as well as noise exposure, apoptotic cell death has been observed in the cochlea. In general, in such models, treatment with a cell permeant type of glutathione monoethyl ester (GSHe) was shown to be protective, further bolstering the notion that apoptosis is an important outcome in cochlear injury from ROS (Kopke et al. 1997; Ohinata et al. 2000).

4.4.5 Oxidant Stress in Aminoglycoside Toxicity

Aminoglycosides are a large family of microbial protein synthesis inhibitors that have seen wide use as antibiotics. They include drugs such as amikacin, arbekacin, gentamicin, kanamycin, and neomycin. Of these, gentamicin and kanamycin have been particularly implicated in enhancing the generation of hydroxyl radicals in cochlear explants (Clerici et al. 1996). Published reports have shown that these compounds likely produce ROS by acting as iron chelators (Song and Schacht 1996; Priuska and Schacht 1997). This is underscored by the demonstration by several

investigators that both iron chelators and ROS scavengers have proven to be effective moderators of aminoglycoside-induced injury (Song and Schacht 1996; Priuska and Schacht 1997). A contrasting argument (e.g., by Basile et al.) has implicated overstimulation by *N*-methyl-D-aspartate (NMDA) by aminoglycosides as the basic mechanism of toxicity in the cochlea (Basile et al. 1996). These authors were able to attenuate hearing loss and hair cell damage by the use of NMDA antagonists demonstrating that aminoglycoside toxicity is excitotoxic in part. Indeed, glutamate release is also a trigger for ROS generation which fits with this alternate picture of NMDA overstimulation (Khan et al. 2000). The role of oxidative stress in aminoglycoside ototoxicity and the use of antioxidants as therapeutics are described in detail in Chap. 10 by Rybak.

4.4.6 Cisplatin Ototoxicity and ROS

Cisplatin [cisplatinum, *cis*-diamminedichloridoplatinum (CDDP)] is a commonly used cytotoxic drug used in cancer chemotherapy. It acts by cross-linking DNA and triggering apoptosis, preferentially in malignant cells. The drug remains useful despite its known toxicity toward the kidney, nervous system, and cochlea (Ravi et al. 1995). Cochlear toxicity manifests as tinnitus followed by hearing loss above 4 kHz (Rybak 1981). Such ototoxicity is often bilateral and tends to correlate with the frequency of cisplatin administration (Powis and Hacker 1991).

The evidence for the involvement of ROS includes observations in cochlear explants of free radicals by electron paramagnetic spin spectroscopy following cisplatin exposure (Clerici et al. 1996). Further, ROS-induced cochlear damage was noted to be accompanied by an accumulation of malondialdehyde, a well-established marker of ROS-related cellular damage (ensuing from the oxidation of polyunsaturated fatty acids), a key marker of ROS-induced membrane damage, as well as a decrease in GSH following exposure to cisplatin and decreased GPx and reductase activity with an increase in SOD and catalase activity in the inner ear (Ravi et al. 1995; Kopke et al. 1999). In addition, ROS have also been detected inside cisplatin-exposed hair cells using vital dyes and confocal microscopic imaging (Ravi et al. 1995; Kopke et al. 1999). Finally, in certain experiments, when the cochlea was observed in the presence of buthionine sulfoximine (a potent GSH synthesis inhibitor), auditory hair cell loss was accentuated (Kopke et al. 1999). The role of oxidative stress in cisplatin ototoxicity and the use of antioxidants as therapeutics are described in detail in Chap. 11 by Laurell.

4.4.7 ROS in Cochlear Senescence

Age-related hearing loss (ARHL; presbycusis) is the most common hearing deficit in those of advanced age and has become a significant social and public health issue (Huang and Tang 2010). It appears that a lifetime of exposure to noise, genetic

susceptibility, a variety of otologic and systemic disorders, and toxic agents are the main etiologic factors (Gates and Mills 2005). In early phases of presbycusis, a high-frequency hearing loss is seen, and the trouble with consonants such as P, H, G, CH, SH, K, F, S, and TH can begin even with mild hearing later. Later, the individual begins to have difficulty in detecting, identifying, and localizing sound sources. Once hearing loss progresses to the 2–4 kHz frequency range, with vowel understanding also compromised, speech comprehension is further compromised.

4.4.7.1 Free Radicals and Damage to Mitochondrial DNA with Age

In the 1950s, Harman proposed a free radical theory of aging based on evidence that was beginning to implicate ROS-induced subcellular damage, defects, and dysfunction (Harman 1956). Subsequent to this novel and intriguing idea being proposed, Miquel and colleagues advanced the mitochondrial degeneration mechanism of senescence (Miquel et al. 1980, 1992; Miquel and Fleming 1984; Miquel 1991). In summary, Miquel's view is that ROS cumulatively injure the circular mitochondrial genome, and because this is the critical repository of information for the bioenergetics machinery, ATP synthesis and regulation becomes compromised over time (Huang and Tang 2010). Miquel's group was one of the early reporters of the phenomenon of life span extension in laboratory animals whose diet was supplemented with antioxidants (Miquel 2002). Disappointingly, this finding has not extended to nonempirical, real-life settings, unless evidence is limit to *Drosophila* (Peng et al. 2014). However, the underlying mechanisms appear to be valid and reproducible within rodent models, and important studies have shown that murine life spans are measurably lengthened when ROS defenses are bolstered. An excellent example of such a report is from Schriner and his colleagues who found life span increases in mice overexpressing catalase targeted to the mitochondria (Schriner et al. 2005). Equally elegant is that older experiments found that antioxidants had little or no impact on murine life spans. Interestingly, in the classic paper by Kohn, antioxidants were able to eke out an effect if controls were exhibiting compromised survival relative to mean population data (Kohn 1971). It is obvious that this is the primary caveat in these studies since it is unclear if the findings are extendable up the phylogenetic scale.

Specifically, evidence has been found that in aged C57Bl6/J mice, antioxidant mRNA levels decline progressively (Staecker et al. 2001). In addition, McFadden et al. have observed that SOD1 and O^{2-} are also implicated in the pathogenesis of ARHL which can be attenuated by therapeutic strategies directed at these molecules (Staecker et al. 2001). Jiang has found measurable levels of ROS in the senescent cochlea (Jiang et al. 2007). In old rats, investigators have also seen evidence that GSH levels are significantly reduced in the eighth nerve (Lautermann et al. 1997). Researchers have strengthened the mitochondrial DNA damage idea in successfully testing for mutations in the mitochondrial DNA and reporting that these are positive determinants of hearing loss in elderly human subjects (Fischel-Ghodsian et al. 1997). Seidman and colleagues demonstrated that a 4,834-bp mitochondrial DNA

deletion (mtDNA) was present in older subjects with ARHL and went on to identify a 4,977-bp mtDNA defect in archived human temporal bone specimens from patients with ARHL (Bai et al. 1997; Seidman et al. 1997, 2004). Additional mtDNA deficits have also been identified which correlate with ARHL. Examples are the 5,142-bp and 5,354-bp deletions in mtDNA reported by Markaryan et al. (2008). Finally, certain mtDNA haplogroups have been shown to be associated with ARHL (Manwaring et al. 2007). Thus, ample evidence not only implicates ROS in being responsible for subcellular defects and dysfunction in the aging organism but, more specifically, the bioenergetics nexus of the cell, the mitochondrion, appears to be the site where these reactive species, both in the short-term and in a chronic sense, create anomalies that lead to perturbations and loss of function.

4.5 Antioxidants as Therapeutic Agents

When looked upon as a typical biological system, ROS and their associated cellular signaling mechanisms manifest complex and parallel architecture such as is seen in most cellular signaling networks. This picture is compounded by the fact that ROS include many different species with varying dynamics and properties. Thus, it is not surprising that most medical interventions that target ROS have not been successful (Steinhubl 2008). Contributing to this observation is the notion that ROS-based signaling is spread across a relatively narrow range from adaptive to maladaptive (Nathan and Cunningham-Bussel 2013). The disappointment of unsuccessful ROS targeting in human disease as well as in many animal models of human pathology is somewhat balanced by the fact that many successful drugs work by impacting ROS signaling or by sensitizing cells to such signals as well as diminishing ROS generation (Nathan et al. 1981; Liou and Storz 2010; Raj et al. 2011). In the ear, the status of science is similar and we will examine it in some detail below.

4.6 NIHL and Antioxidants

It has to be stated at the outset that there is no FDA-approved drug that is currently available to prevent or treat NIHL (Lynch and Kil 2005). However, many studies in animal models of NIHL have reported robust attenuation of NIHL by agents that diminish or protect from the effects of reactive species (Henderson et al. 1999, 2006; Le Prell et al. 2003; Lynch and Kil 2005). One of the early entrants in antioxidant research for the treatment or prevention of NIHL was the compound allopurinol, a xanthine oxidase inhibitor used to treat hyperuricemia. With systemic injection of allopurinol during and after noise exposure, allopurinol reduced the resulting permanent hearing loss (Seidman et al. 1993). Later data suggested this effect was limited as allopurinol was only able to rescue NIHL if the threshold shift was temporary (TTS) and not when the noise exposure was high enough or long enough to cause a permanent shift in threshold (PTS) as assessed 15–30 days post noise exposure (Franze et al. 2003).

More recently several other antioxidants have been used with interesting results. These include the GSH precursors *N*-acetylcysteine (NAC) and methionine (MET). When injected intraperitoneally, both NAC and MET were shown to be otoprotective (Kopke 2001). It is worth to note there are at least two studies with human subjects in which TTS was reduced by an antioxidant agent, including alpha-lipoic acid (Quaranta et al. 2012) and vitamin B12 (Quaranta et al. 2012). In 2011, Lindblad and colleagues reported that oral administration of NAC was capable of protecting the cochlea from loss of nonlinearity (the peculiar transductive mechanism of the inner ear that makes a wide dynamic range possible) from impulse noise (in this case, soldiers exposed to firearm discharge noise) (Lindblad et al. 2011). In contrast, Kramer et al. and Lin et al. have reported less successful attempts toward otoprotection from NAC administration in humans subject to noise (Kramer et al. 2006). Lin and colleagues also tested NAC as an otoprotectant after industrial noise exposure (Lin et al. 2011). This was a prospective, double-blind trial in male industrial workers exposed to 88–89 dBA noise with the intervention group receiving 1,200 mg a day of NAC for 2 weeks. This trial did not reveal any benefit from the use of the antioxidant. In both the Kramer and Lin studies, certain limitations could have disguised an effect. In the former, subjects were recruited who had been exposed to noise in a nightclub. Here, the consistency of exposure and wide variance in sensitivity of the subject could have been a confounder. It has been argued by Le Prell and her colleagues [who wrote an excellent review of the topic; (Le Prell et al. 2012)] that similar evaluations undertaken in a laboratory environment and with calibrated equipment might circumvent the methodological limitation and reveal an effect. In Lin's report, Le Prell further argued that with a test–retest reliability of about 5 dB, an effect of attenuation in threshold shift (as reported by Lin et al.) of less than 3 dB would easily obscure the results. In addition, because these trials are relatively limited in scale, results can only be extrapolated with caution. Many commentators and investigators have expressed the need for larger human trials with potential otoprotectants like NAC (Scheck 2012). Several new studies have consolidated the reputation of NAC in protecting from NIHL. Dr. Kopke's group in Oklahoma City has begun to report on an intriguing combination intervention where NAC is administered to noise-deafened animals along with a spin trap agent called HPN-07. Spin trap compounds were first noted to display synergism with NAC in attenuating NIHL by Choi et al. (2008). In the Kopke laboratory, 21 days of OBN (octave band noise) exposure was used to cause threshold shifts, and NAC was administered with the butylnitrone spin trap agent HPN-07. The report documented reduced ABR (auditory brainstem response) threshold shift in treated animals, lower DPOAE (distortion product otoacoustic emissions) shifts, and marked diminishment of hair cell loss in the basal cochlea (Lu et al. 2014).

D-methionine (a widely used free radical scavenger) has also often been used by investigators to assess its efficacy as an otoprotectant in NIHL. For example, Sampson and colleagues demonstrated that 400 mg/kg IP dosing of D-methionine was able to reverse noise-induced (4 kHz OBN; 100 dB SPL) oxidative stress in the mouse cochlea (measured as lipid peroxidase, SOD, and catalase activity) (Samson et al. 2008). Over many years, Campbell and colleagues have exhaustively detailed

the protective role of D-methionine in the inner ear. In early work they reported that D-methionine attenuated damage to the stria vascularis in a cisplatin ototoxicity model (Campbell et al. 1999). In rats, her group demonstrated that this enzyme protected against cisplatin-induced ototoxicity, in part, by increasing the levels of antioxidant enzymes in the inner ear (Campbell et al. 2003). More recently, this laboratory has also shown that D-methionine can protect against noise-induced cochlear injury (Campbell et al. 2007) In chinchillas rendered permanently deaf with noise-induced hearing loss, NAC and D-methionine together showed effective protective synergy, while the two compounds acting alone did not (Clifford et al. 2011). More recently, dose-dependent protective effects of D-methionine have been reported in guinea pigs with NIHL (125–15 kHz, broad band white noise at 105 dB SPL) when the compound was administered at doses of 200, 400, and 600 mg/kg IP (Lo et al. 2013). At around the same time, another report from a laboratory that has generated a significant volume of D-methionine literature in NIHL described that when D-methionine was administered prior to noise exposure in chinchillas, hearing thresholds (measure by ABR recordings) were significantly improved (Claussen et al. 2013).

Other antioxidants have also been tested as potential otoprotectants in NIHL. These include vitamins A, C, and E, α-lipoic acid, and magnesium. Le Prell and colleagues published reports a few years ago showing that when more than one free radical scavenger was used in noise-induced hearing deficits in male guinea pigs, ABR thresholds were preserved. Due to variance, statistical power, and the designed mean intergroup difference the investigators had set for themselves, histological changes from noise trauma were less obvious between controls and treatment groups, and failed to reach statistical significance (Le Prell et al. 2007). Intriguingly, α-lipoic acid has also been shown to reduce carbon monoxide-induced potentiation of NIHL in rats (Pouyatos et al. 2008). Heinrich et al. also published data in a guinea pig NIHL model demonstrating that ascorbic acid treatment was able to reduce hearing thresholds after exposure to 90 dB SPL noise for 1 h. The authors also presented data on NO activity in the cochlea with ascorbic acid treatment. However, it was challenging to critique this report overall because the researchers failed to provide adequate detail of their NIHL paradigm (Heinrich et al. 2008). Le Prell has also compelled the notion of otoprotective dietary intervention on the principle of antioxidant interventions in NIHL. In their 2011 study, Le Prell et al. subject CBA/J mice to 8–16 kHz OBN noise for 2 h. Noise-naïve controls and noise-exposed animals were subject to ABR testing and micromorphometric evaluation of the cochlea with or without customized dietary manipulation (normal mouse chow supplemented with β-carotene, vitamin C, vitamin E, and magnesium). The study revealed a significant beneficial impact of the supplemented diet on ABR thresholds after noise exposure. While hair cell survival was not statistically improved, the number of type II fibrocytes in cochlear lateral wall was greater in animals fed the supplemented diet, and cell density of this type was found to be comparable to mice that had not been exposed to noise at all. There has been evidence from other studies that microstructural preservation of the type evident in these experiments correlates with ABR threshold preservation. Therefore, this finding

has interesting implications (Mizutari et al. 2008; Le Prell et al. 2011). Other important work in subsequent years has also validated the role of α-lipoic acid as an antioxidant/otoprotectant in NIHL, either alone of as part of an enhanced diet in human studies. Prominent among these is Quaranta et al. who reported findings in normal human subjects who had been induced to have TTS after exposure to 90 dB of 3 kHz pure tone sounds for 10 min. Controls who received only the noise were compared with those who were subject to noise as well as preexposure oral treatment with α-lipoic acid (600 mg) and a third group who were given the same dose of the compound for 10 days prior to noise exposure. ABR thresholds of this third group (long-term preexposure prophylaxis) as well as the amplitude change of transient evoked otoacoustic emissions (TEOAEs) were lower in the long-term supplementation group compared to the other two groups (naïve controls and single-dose groups) (Quaranta et al. 2012). Additional signaling systems in the cochlea also mediate ROS-induced effects on cochlear physiology. For example, our work with the stilbenoid, resveratrol (a compound touted for its health benefits especially those from its presence in red wine), has shown that it attenuated the expression of cyclooxygenase-2 (COX-2) and concomitant ROS formation in the rat cochlea and that it reduces [it is more accurate] hearing threshold shift from acoustic trauma (Seidman et al. 2003, 2013). The basis of stable adhesion of leucocytes to the vascular endothelium is thought to be mediated at least in part by the expression of intercellular adhesion molecule-1 (ICAM-1), an inducible endothelial adhesive protein that serves as a counter-receptor for β_2-integrins on leukocytes. Interaction of ICAM-1 with β_2-integrins enables leukocytes to adhere firmly to the vascular endothelium and subsequently to migrate across the endothelial barrier. There is evidence that ICAM-1 expression, in general, can also promote ROS generation ubiquitously (Rahman and Fazal 2009). In view of this notion, our group also tested the possibility that immunochemical blockade of the ICAM-1 receptor might prove protective in NIHL via the deflation of ROS synthesis. We reported that intravenous administration of ICAM-1 antibody to Fischer rats successfully reduced the severity of hearing loss from broad band noise exposure (Seidman et al. 2009).

These are all compelling studies and have strongly advocated for expanding the evaluation of these promising antioxidants in human trials. Some limitations of animal studies apply in these examples and may be summarized as routes of administration that may not be possible in human and/or clinical settings and high experimental doses which pose challenges with respect to translation to the bedside. Finally, the bioavailability of antioxidants is varied and will need to be carefully determined in future studies.

4.6.1 GPx

In guinea pigs, the use of ebselen (which is a GPx mimic and inducer), when administered by gavage (mimicking the per oral route), has proven somewhat protective in TTS and PTS models of NIHL (Pourbakht and Yamasoba 2003; Lynch et al. 2004).

Such studies are consistent with, and build on earlier evidence that GPx activity is notably decreased in NIHL and that engineered deletion of the GPx1 gene makes animals susceptible to NIHL (Ohlemiller et al. 1999, 2000). Specifically, Ebselen has been shown to diminish the severity of NIHL in rodent models with oral dosing (Pourbakht and Yamasoba 2003; Lynch et al. 2004). It has been postulated that because ebselen has been shown to block Cyt c release from the mitochondrion in cells that are stressed to the point of triggering the apoptotic cascade, the compound might exert beneficial effects on the cochlea via this mechanism as well (Boireau et al. 2000; Namura et al. 2001). Indeed, studies have compellingly demonstrated that ebselen can mitigate NIHL via its mimicry of GPx induction. Kil et al. found that GPx1 was a highly expressed isoform of GPx in the cochlea with concentrations in the rat organ of Corti, the spiral ganglion, the stria, and the spiral ligament. These investigators induced NIHL with 5 h of 113 dB noise (4–16 kHz) and administered ebselen at a dose of 4 mg/kg IP. The drug was able to reduce OHC loss secondary to noise exposure, minimize swelling of the stria, and boost GPx1 levels in the stria compared with controls (Kil et al. 2007). Yamasoba et al. have also published work that underscores ebselen's protective role in NIHL. Their 2005 report looked at guinea pigs who had been exposed to 115 dB of OBN centered at 4 kHz. In these experiments, ebselen eliminated ABR threshold shifts and significantly tempered microanatomic changes from noise in the cochlea (swelling of afferent dendrites underneath IHCs (Yamasoba et al. 2005)). Recently, both phase I and II clinical trials with ebselen have completed and are being evaluated (Sound 2014; Lynch and Kil 2009).

4.6.2 Magnesium

Nearly 20 years ago, Attias et al. reported the finding that oral supplementation with magnesium (equivalent to 6.7 mM of Mg aspartate) was sufficient to afford partial protection from PTS 1 week after noise exposure (Attias et al. 1994). In contrast, a weak correlation was published between current serum levels of this mineral and the measured PTS in previously noise-exposed military combatants in a study by Walden and colleagues (Walden et al. 2000). This was interesting because on the one hand, magnesium supplementation appeared to offer protection in soldiers in terms of the severity of TTS. In a laboratory-based human subject study, no significant changes were noted in serum Mg levels between noise-exposed individuals and controls (Attias et al. 2004). Bearing in mind that Mg levels in the serum can be measured with difficulty and with less than desirable reliability, the relationship between magnesium supplementation and protection from NIHL, while provocative, needs to be investigated further. In fact there is now an extensive body of literature on the effects of Mg in the prevention of NIHL (for review, see Le Prell and Bao 2011). The mechanism of action of magnesium is also of interest as it is a potent glutamate antagonist and can mitigate glutamate excitotoxicity which may be implicated in NIHL (Mayer et al. 1984; Smith et al. 1989; Clerc et al. 2013).

4.6.3 N-*Acetylcysteine*

Continuing with the theme of military personnel who are exposed to noise, weapon training in the Marine Corps has been used as a test bed to determine the efficacy of NAC as an otoprotectant from noise. In 2003, a significantly sized trial by the US Department of the Navy enlisted 566 marine recruits who were undergoing weapon training with standard issue assault rifles. This was a randomized study with the treatment group receiving 900 mg of NAC with each meal through the length of the study. Possibly because all participants (experimental as well as control) were required to wear hearing protection, only about a third experienced measurable hearing loss. Of those who did, NAC was shown to have about a 25 % protective effect (modest by any standard) (Dolgin 2012). In contrast, under somewhat different circumstances, NAC was not able to exhibit protection in subjects who were exposed to loud sounds in a nightclub that had resulted in an average 14 dB TTS at 4 kHz (Kramer et al. 2005). In aggregate, the evidence does not clearly support NAC as a reliable protectant in NIHL.

4.6.4 *Iron Chelators*

A variety of iron chelating compounds can protect the cochlea by offering less iron to be available to react with H_2O_2, thereby limiting the Fenton reaction (iron-catalyzed hydrogen peroxide to yield hydroxyl radicals has been called Fenton's reaction). In this class of compounds, deferoxamine (DFO) and dihydroxybenzoate (DHB) have both been used with some success in attenuating NIHL (Yamasoba et al. 1998). Iron-dependent ototoxic mechanisms in cisplatin-induced cochlear damage have also been investigated. When the iron chelator 2,2′-DPD (dipyridyl) was employed in guinea pig cochlear explants that had been exposed to cisplatin, significant rescue from cell death was observed (Rybak and Kelly 2003).

4.6.5 *Trophic Factors*

Brain-derived neurotrophic factor (BDNF) and glial cell-derived neurotrophic factor (GDNF) have also been used to rescue the cochlea from noise-induced injury (Shoji et al. 2000). The exact mechanism of these factors through which they purportedly provide protection, remains unclear. It is assumed, though not proven that they might have a role in upregulating antioxidant enzyme levels or alternatively in suppressing apoptotic triggers in the hair cells or spiral ganglion neurons (Jackson et al. 1990a, b; Perez-Polo et al. 1990; Pan and Perez-Polo 1993).

Overall, NIHL remains a significant public health problem. In some ways, an advancing technological society such as in the western hemisphere, the ubiquitous

presence of environmental noise (e.g., industrial noise, and transportation noise) and occupational noise exposure (e.g., military, and civil aviation) challenges us to explore ways and means to ameliorate its health effects, including that on hearing. Much empirical work has identified the details of noise-induced hearing deficits. While many promising techniques have been discovered to protect the cochlea from the effects of noise, a single-agent protective regimen remains elusive. It is possible that multi-action modalities of treatment and prevention (such as synergistically acting drugs) will prove to be the mainstay of hearing protection from acoustic overstimulation. However, it is likely that significant advances will have to be made to achieve these goals.

4.7 The Use of Antioxidants in Aminoglycoside Toxicity

Rybak has described two strata of otoprotection in the context of aminoglycoside toxicity in the cochlea. These he has termed "upstream" (intervention that prevents the initial stages of lipid peroxidation at the hands of ROS) and "downstream" (interventions that can slow or reverse the process of cell death in the cochlea) (Rybak and Whitworth 2005). Between 1999 and 2000, investigators at a military medical university in China and the University of Michigan undertook a prospective, randomized, double-blind trial of aspirin supplementation in patients who were being treated with gentamicin for acute infections. This trial provided cogent evidence that an antioxidant can prove very useful in instances of aminoglycoside-induced ototoxicity. The trial showed that only 3 % of aspirin users suffered the consequence of hearing loss from gentamicin exposure. while hearing loss was noted in 13 % of those who were administered gentamicin with placebo (Sha et al. 2006; Chen et al. 2007).

4.8 Antioxidant Strategies in Aminoglycoside Ototoxicity: Upstream Protection

Several different drugs or compounds have been employed in attempts to block or mitigate early events in ROS-mediated aminoglycoside toxicity. These include vitamin E, D-methionine, α-lipoic acid, and ebselen (Takumida et al. 1999; Sha and Schacht 2000; Fetoni et al. 2003). Promising results were also recently reported for a combination of antioxidants including several vitamins and magnesium (Le Prell et al. 2014). In addition, iron chelators like deferoxamine have also been used with modest success (Dehne et al. 2002). More exotic ideas have also been tested, again with varying success, and these include modulating the role of NF-κB in the apoptotic cascade by using salicylate and 2,3-dihydroxybenzoate (Jiang et al. 2005), as well as an emerging report on nutrient supplementation in gentamicin ototoxicity

(Le Prell et al. 2014). Polony and colleagues have found promising results with rasagiline (a selective monoamine oxidase type B inhibitor used in early Parkinson's disease therapy) in aminoglycoside toxicity. The choice to test this drug was based on its known anti-apoptotic properties (by interfering with mitochondrial-dependent apoptotic signaling). In this paper a mouse model of kanamycin ototoxicity was used as a test bed, and the data demonstrated that at a dose of 3 mg/kg SC, rasagiline mitigated kanamycin-induced shifts in ABR thresholds in a dose-dependent manner. The investigators further explored potential mechanisms of this effect and found that the drug enhanced action potential-evoked dopamine release in the mouse cochlea (Polony et al. 2014). Chen et al. have also tested the mammalian peroxiredoxin, Prx3, as a mechanistic marker in a CBA/J mouse model of kanamycin toxicity. In their study, Prx3 was introduced into the cochlear explants via siRNA delivery with a hole drilled into the structure close to the round window via a microtube. The authors tested for the generation of free radicals in the hair cells in response to aminoglycoside exposure by immunofluorescent techniques. They then monitored changes in Prx3 expression in the hair cells in the context of gentamicin and kanamycin-induced hair cell loss. The data showed increased Prx3 expression in OHCs after about a week of kanamycin exposure. With loss of hair cells, there was a concomitant decrease in Prx3 levels as well. Further, these investigators used aminoglycoside-induced hair cell loss to test if Prx3 levels were correlated with antioxidant-mediated cell survival (using 2,3-dihydrobenzoate [DHB]). Interestingly, DHB treatment in kanamycin-induced cochlear injury also preserved the level of expression of Prx3 (Chen et al. 2013). Coffin et al. have also recently demonstrated that cell death pathways in hair cells in ototoxicity from aminoglycosides are more specific than previously assumed. For example, in Coffin's zebra fish lateral line model of ototoxicity, neomycin, gentamicin, and cisplatin induced or triggered a distinct subset of the programmed cell death machinery. This was evident from the differing response this model exhibited when exposed to specific protective interventions. A proteasome inhibitor protected the hair cells from either aminoglycoside or cisplatin toxicity. D-Methionine, on the other hand, protected from gentamicin and cisplatin effects but not from neomycin toxicity (Sha and Schacht 2000; Campbell et al. 2007; Coffin et al. 2013).

Neurotrophic growth factors have been tried, and these have displayed their ability to bolster antioxidant enzyme activity, reduce the formation of NO [thereby attenuating the involvement of RNS], and increase the anti-apoptotic Bcl-2 proteins in the noise-exposed cochlea (Rybak and Whitworth 2005). Glucocorticoids like dexamethasone have been used to protect isolated OHCs from aminoglycoside toxicity; however, the exact mechanism is unclear. Again, it is presumed that steroids function to inhibit NO and diminish ROS synthesis (Rybak and Whitworth 2005). In addition, certain experimental compounds designed to augment endogenous antioxidant enzymes have seen recent favor, and they include the SOD mimetic M40403 which has exhibited protection of organotypic OHC culture exposed to gentamicin (Nicotera et al. 2004). Interestingly, in a guinea pig model of aminoglycoside toxicity, adenoviral vectoring of SOD1, SOD2, and catalase was partially able to protect the inner ear from toxicity of aminoglycosides (Kawamoto et al. 2004).

Non-pharmaceutic modalities have also been employed in aminoglycoside ototoxicity research. These include "cell toughening" (preexposing the cochlea to low levels of toxic stress to eventually tolerate higher levels of the drugs) has been shown to upregulate the level of antioxidant enzymes in the cochlea and specifically in the guinea pig; the use of amikacin in this manner has been successful (Jacono et al. 1998; Ding et al. 2003). While loop diuretics are known to display synergism with aminoglycosides with regard to ototoxicity, delayed administration of ethacrynic acid has been shown to reduce the level of aminoglycosides in the perilymph with concomitant reduction in cochlear damage (Ding et al. 2003).

4.8.1 Antioxidant Strategies in Aminoglycoside Ototoxicity: Downstream Protection

With the emerging possibility that apoptosis may not be irreversible once initiated, investigators have tested methods to block early apoptosis promoters such as GTPases, Rho, and Rac. One such candidate is toxin B of *Clostridium difficile* (*C. difficile*) which has been reported to provide dose-dependent protection from aminoglycosides (Bodmer et al. 2002). Further, the inhibition of the JNK signaling cascade has also been shown to attenuate aminoglycoside effects in the cochlea (Abi-Hachem et al. 2010). Another similar compound, D-JNK-1, has shown positive results in neomycin toxicity (Wang et al. 2003b; Minami et al. 2004).

Further downstream, blockade of the release of Cyt c from the mitochondrion with the tetracycline antibiotic minocycline has also reportedly yielded positive data in vitro (Corbacella et al. 2004). It has also been shown that caspase inhibitors combined with a p38 MAPK inhibitor afford synergistic protection in models of gentamicin toxicity (Wei et al. 2005).

In summary, while aminoglycoside antimicrobials are very useful as therapeutic agents against serious gram-negative infections in clinical practice, their potential for cochlear injury and their ability to sensitize the inner ear to noise-induced damage make them agents that must be used with great care. About a third of subjects who receive aminoglycosides will experience adverse effects and their slow clearance from cochlear fluids compounds their toxic profile (Campo et al. 2013). These issues are examined and discussed in much greater detail in Chap. 12 of this volume.

4.9 Antioxidant Strategies in Cisplatin Ototoxicity

Cisplatin's clinical usefulness is seriously compromised by its associated nephrotoxicity, neurotoxicity, and ototoxicity. Over the previous few decades, more and more evidence has emerged that cisplatin ototoxicity is mediated via the overproduction of or underprotection from ROS (Clerici et al. 1996). In animal models

cisplatin administration has been proven to deplete GPx, SOD, catalase, and glutathione reductase (Rybak et al. 2000). The isoform of NADPH oxidase (a membrane bound complex and important source of superoxide), NOX3, is preferentially expressed in the inner ear, and in this location it is also an important source of ROS synthesis. Studies in non-cochlear tissue have implicated NOX3 as being responsible for superoxide production in response to cisplatin (in HEK293 cells) (Banfi et al. 2004). This implies the possibility that the enzyme could be tightly targeted to protect from cisplatin toxicity (Rybak and Whitworth 2005). It is not inconceivable that specific inhibition of Nox3 might achieve protection in the cochlea, and in other contexts, several such compounds are being investigated (Schramm et al. 2012). Antioxidant strategies in the case of cisplatin, like aminoglycosides described above, can also be viewed in upstream and downstream contexts.

With cisplatin toxicity, pharmacologic compounds containing thiol groups have been widely tested based on the logic that thiols are potent reducing agents to countermand the impact of oxidative stress. Thus, sodium thiosulfate, D- and L-methionine, diethyldithiocarbamate, methylthiobenzoic acid, lipoic acid, NAC, tiopronin, glutathione ester, and amifostine have reduced cisplatin ototoxicity (Rybak and Whitworth 2005). Sodium thiosulfate perfusion into the guinea pig cochlea, for example, was shown by Wang and colleagues to protect from cisplatin-induced damage (Wang et al. 2003a). When rats were pretreated with D-methionine and then exposed to cisplatin, auditory thresholds and hair cell populations were preserved and antioxidant enzyme depletion was minimized (Campbell et al. 2003). In hamsters, similar methods with amifostine also proved effective (Church et al. 2004).

When rats were pretreated with an iNOS inhibitor (recalling that NO is a key source of RNS molecules) called aminoguanidine, cisplatin-induced cochlear damage was significantly ameliorated, malondialdehyde production was reduced, and ABR thresholds were less severely impacted (Kelly et al. 2003). As in the case of antioxidant strategies employed for aminoglycoside toxicity, allopurinol and ebselen both have proven useful in experiments with cisplatin. In one study as an example, OHCs and auditory thresholds in rats were preserved when these compounds were therapeutically used in cisplatin exposure (Lynch et al. 2005).

Evidence also suggests that antioxidant defense mechanisms in the cochlea are likely operational via adenosine receptors (ARs) or at least that these receptors play a critical role (Rybak and Whitworth 2005). Therefore, when cisplatin is applied to the round window in the chinchilla, the A1AR isoform of the ARs expressed in the cochlea is noted to be upregulated. Further, when the A1AR agonist R-PIA or CCPA was used for pretreatment, fewer cochlear hair cells were damaged from cisplatin use, and ABR thresholds were better preserved (Ford et al. 1997).

In guinea pig studies when the caspase-3 inhibitor z-DEVD-fmk and the caspase-9 inhibitor z-LEHD-fmk were perfused into the cochlea, apoptosis was effectively blocked in response to cisplatin exposure and hair cell loss, and ABR threshold elevations were mitigated (Wang et al. 2004). The p53 inhibitor pifithrin-α was also found competent in protecting from cisplatin-induced damage in organotypic OC cells (Whitworth et al. 2004).

Cisplatin is clearly an important antineoplastic agent that has been used with great success in the battle against malignant solid tumors originating from various organs. It is both nephrotoxic and ototoxic. While its effect on the kidney can be modulated by paying to attention to hydration and water balance, the ototoxic component is much more challenging to counteract or prevent. This phenomenon is further complicated by the concern that antioxidant-based strategies to protect the ear from the drug might inadvertently enhance tumor cell survival (Oishi et al. 2012). Chapter 11 of this volume is dedicated to exploring cisplatin toxicity in much greater detail.

4.10 Antioxidants as Therapeutic Modalities in Presbycusis

The incidence of presbycusis increases with each decade of life, and epidemiological data shows that of individuals who are in their 70s, more than half exhibit this type of hearing impairment (Gates and Cooper 1991; Helzner et al. 2005; Gopinath et al. 2009). The use of antioxidant strategies to combat presbycusis has had mixed results as reviewed below.

4.10.1 Testing Antioxidants in Presbycusis

Seidman et al. (2000) reported that vitamin C, vitamin E, and melatonin, all known to possess antioxidant properties, attenuated hearing loss in F344/NHsd rats who were allowed to age normally (Seidman et al. 2000). These efforts also included testing the compound acetyl-L-carnitine (ALCAR) and α-lipoic acid based on the ability of these compounds to improve mitochondrial bioenergetics in NIHL. Indeed ALCAR was effective in presbycusis, again in a rat model (Seidman et al. 2000). However, enthusiasm was dampened when Bielefeld et al. could not reproduce ALCAR's protective effects in the same species (Bielefeld et al. 2008). Seidman et al. have also reported success in presbycusis by using lecithin (a plant-derived phosphatidylcholine that can enhance membrane antioxidant defenses). In these experiments, 6 months of oral supplementation in F344 rats attenuated the progress of presbycusis when the animals were permitted to age from 18 months onward (Seidman et al. 2002).

Like ALCAR, investigators have also used NAC with the hope that because this compound operates, in part, as an antioxidant in NIHL-based studies, it might show benefits in presbycusis as well. However, NAC has not shown promise in presbycusis as underscored by studies involving both C57/Bl6 mice and F344 rats (Davis et al. 2007; Bielefeld et al. 2008). In the context of presbycusis, the role of apoptotic mechanisms in an aging organism has been evaluated in transgenic mice by Someya and colleagues. In their comprehensive set of experiments, the pro-apoptotic factor Bak was assessed for its contribution to presbycusis. C57BL/6J $Bak^{-/-}$ were used and compared with matched wild-type controls. ABR thresholds in middle-aged $Bak^{-/-}$ animals were significantly lower than WT at low frequencies. However, no

differences were noted for thresholds at middle and high frequencies (Someya et al. 2009). This was suggestive of the criticality of Bak to the pathogenesis of aging in the cochlea. In contrast, it was reported by this group that no significant differences were seen in ABR thresholds between WT and Bax-deficient mice. Someya also investigated whether primary cochlear cells devoid of Bak ($Bak^{-/-}$) could be shown to resist oxidative stress-induced cell death from paraquat exposure (a toxic herbicide that acts via ROS generation in plant and animal tissues). In this set of experiments, primary cochlear cells derived from WT and $Bak^{-/-}$ mice were examined under the influence of paraquat, revealing that while the herbicide reduced cell viability in a dose-dependent manner, in cells that lacked Bak ($Bak^{-/-}$), there was appreciable resistant to cell death compared with the WT (Someya et al. 2009). In another interesting study, Heman-Ackah et al. have shown that a combination diet with six different antioxidants fed to C57BL/6 mice modeled to have presbycusis, ABR thresholds were decreased compared with controls with a strong statistical effect (Heman-Ackah et al. 2010). These finding have been tempered by the findings from Dr. Schacht's laboratory (Sha et al. 2012) where CBA/J mice were monitored from young to through 24 months of age with an intervention group maintained on an antioxidant diet (vitamins A, C, and E as well as L-carnitine and α-lipoic acid). These experiments did not show any differences in hearing thresholds between animals that received the supplementation and those who did not. The authors have examined their findings with exceptional candor. In view of several past studies (some mentioned here as well as those from their own 2010 report) that did show a benefit from such intervention, the concluding remarks point to that while the authors are not refuting the broad benefits of an antioxidant-rich diet on the aging organism, for normally maintained animals (i.e., those with no prior deficiencies), supplementation was not proven to be effective (Willot and Schacht 2010; Sha et al. 2012). Spankovich and Le Prell have recently made the important analysis of the topic by looking at human subjects in a cross-sectional study utilizing a "healthy eating" index and hearing data in adults with ages between 20 and 69. The cohort was derived from the National Health and Nutrition Examination Survey (NHANES; 1999–2002). After the researchers controlled for age, race, ethnicity, sex, education, diabetes, and noise exposure, a strong negative correlation was found between an index score coinciding with a high-quality diet (high in antioxidants) and hearing thresholds at higher frequencies with no effect seen at lower ones (Spankovich and Le Prell 2013).

4.10.2 Presbycusis Research in Animals Lacking Antioxidant Defenses

An intriguing body of work has recently emerged in transgenic mouse models that lack SOD1, a critical element in the cochlear ROS defense. Keithley and colleagues demonstrated that in these rodents, presbycusis was significantly accelerated as the mice grew older, and in them microanatomic changes were marked and dramatic.

These included severe spiral ganglion cell loss and reduced size of the stria vascularis (Keithley et al. 2005). Furthermore, McFadden et al. also reported that OHC loss was accelerated when SOD1 was genetically deleted (McFadden et al. 1999).

In keeping with these findings, attempts have been made to overexpress SOD. However, these efforts have not been successful with respect to protection from presbycusis. For example, Coling et al. and Keithley et al. have both reported failed experiments in recent years (Coling et al. 2003; Keithley et al. 2005). Interestingly, genetic knockout of the GPx gene in mice has also been described by Ohlemiller to exacerbate, much like SOD1 depletion, age-associated hearing impairment. Thus, the time is ripe to systematically tease out the consequences of genetic manipulation of antioxidant defenses in the context of presbycusis (for a comprehensive review of molecular targets in bolstering antioxidant defenses, see Chaps. 13 and 14).

4.11 Conclusions

The cochlea can be assaulted by a variety of stressful and toxic impacts. These range from high intensity noise, drugs and toxins, and the process of aging to name the commonest types. While the organ is capable of defending itself against these insults, they are by no means indefatigable or impenetrable.

A mounting body of work has implicated reactive oxidative species as members of a complex set of signaling systems in the cells of the cochlea that trigger pernicious consequences leading to functional impairment as well as programmed death. The ensuing loss of hearing has become one of the leading public health issues of the modern era where the faculty of hearing is increasingly valued in the setting of a technological civilization.

Because reactive chemical species are part of a bewildering array of interconnected systems in the cell, it is not surprising that attempts to manipulate their function with the object of battling pathology in general, and in the context of this discussion, hearing loss in particular, have proven challenging. It is becoming obvious that the effort to prevent and treat hearing loss must continue forward along with several definable strategies. These include more detailed evaluation of the biochemical and molecular mechanisms of cell death in the cochlea, the biochemistry of oxidants and antioxidants relevant to the cochlea, and to exploit advances in computation technology and genetics in designing more effective treatment strategies in hearing loss, whether from toxins, noise-related injury, or aging.

It is clear that these advances will take place and that the field will advance exponentially. The challenge of research economics appears to be an immediately identifiable obstacle, but this is a sociopolitical issue that always remains in flux. This problem notwithstanding, the scientific advances in recent years have been very encouraging. In NIHL, dietary and pharmacologic interventions have shown promise. Some examples are the groundbreaking work done with β-carotene, antioxidant vitamins, magnesium, L-NAC, and D-methionine. Experts have estimated that the

first putative oral otoprotectants in NIHL might emerge within the next decade (Oishi and Schacht 2011). Efforts are also underway to identify susceptibility genes related to NIHL. If successful in the next few years, these expected discoveries will help identify biomarkers for NIHL sensitivity. In addition, discovery of these genetic loci might even allow for the development of genetic therapies to combat NIHL and perhaps even reverse it once it occurs (Sliwinska-Kowalska and Davis 2012). In presbycusis, a large body of work has identified several key mechanisms of hearing loss that include age-related diminution of antioxidant defenses, mitochondrial genetic deletions, and hereditable traits. Here again, antioxidant therapies have shown promise even though the success of these methods appears to depend on factors such as the type and dose of the antioxidant, the timing and duration of therapy, and the species and strain receiving the intervention (Yamasoba et al. 2013). It is widely held that to prove the value of therapies that slow or delay the onset of presbycusis (such as antioxidants), large clinical trials will be needed, and several authorities in the field have pushed for such research very strongly in recent years. In the pursuit of developing modalities to treat or prevent drug-induced ototoxicity (such as from aminoglycosides and cisplatin), promising approaches have included antioxidants as well (e.g., vitamin E). In the case of cisplatin, a few protective compounds were tested, but the problem of their interfering with cisplatin's tumor fighting capacity was a prominent roadblock (such as some sulfhydryl-containing compounds) (Rybak and Whitworth 2005). Another such agent was amifostine which showed otoprotective effects in hamsters (Church et al. 2004) and has had mixed results in clinical trials with disappointing results in some (Marina et al. 2005; Sastry and Kellie 2005) and success in others (Fouladi et al. 2008). Another more recent promising candidate is cimetidine (the H_2-receptor antagonist used in peptic ulcer disease). Cimetidine has shown quite promising results in animal studies as well as in human tumor cell lines (Schacht et al. 2012). In the case of aminoglycoside antibiotics and their ototoxic problem, antioxidants like vitamin E, *N*-acetylcysteine, and salicylates have all been used with some positive results (Schacht et al. 2012). Thus, it is fair to say, rapid advances in combating cochlear injury regardless of cause and etiology. Many strategies are under development based on better mechanistic insights. Several are in trials that show promise with good efficacy and acceptable or exceptional safety margins. Technological advances in the coming years will likely reduce the incidence of acquired hearing loss to a level where health and economic impacts will become negligible.

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Part III
Epidemiology of Hearing Loss

Chapter 5
Role of Free Radicals in Hearing Loss due to Heavy Metals

Sung Kyun Park

Abbreviations

BAEP	Brainstem auditory evoked potential
CAP	Compound action potential
CAT	Catalase
GGT	γ-glutamyltransferase
GPx	Glutathione peroxidase
GSH	Glutathione
MDA	Malondialdehyde
NADPH	Nicotinamide adenine dinucleotide phosphate
ROS	Reactive oxygen species
SOD	Superoxide dismutase

5.1 Introduction

Heavy metals, such as lead, cadmium, and mercury, are widespread and notable for their toxic effects even at low levels of exposure encountered in the general environment. Lead, mercury, and cadmium are ranked as the second, the third, and the seventh hazards, respectively, that pose the most important potential threat to human health on the 2011 priority list of the US Agency for Toxic Substances and Disease Registry (ATSDR) (www.atsdr.cdc.gov/spl/). Unlike essential metals such as iron

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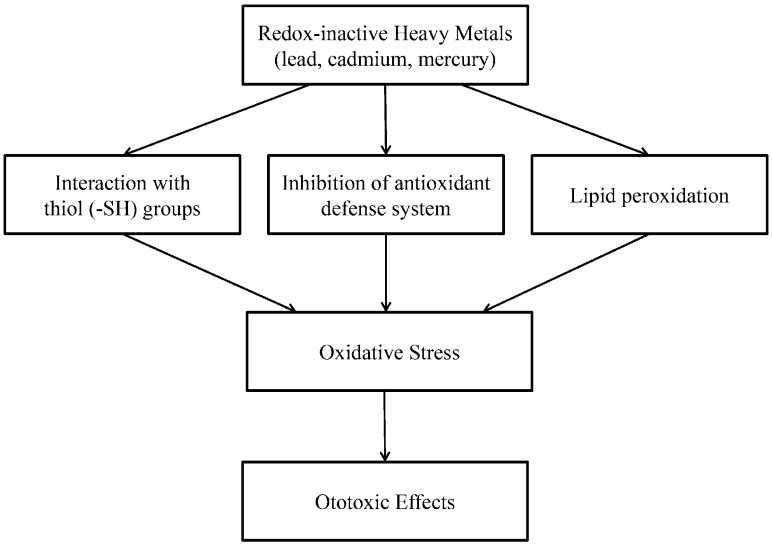

Fig. 5.1 Proposed mechanisms of oxidative stress by redox-inactive toxic metals

and zinc, these heavy metals have no physiological functions in cell homeostasis and have been associated with various pathologies in the kidney and cardiovascular and nervous systems. Although the exact biological mechanisms are poorly understood, oxidative stress induced by exposure to these metals seems to play a central role in their toxicity, including their effects on the auditory system.

Redox-active metals, such as iron and copper, generate reactive oxygen species (ROS) and induce oxidative stress through Fenton-like reactions (Ercal et al. 2001; Jomova and Valko 2011), a process that has been well documented in the inner ear as well (Evans and Halliwell 1999). The toxic heavy metals aforementioned, however, are redox-inactive metals and thus lead to oxidative stress via more indirect mechanisms, which are less understood. Possible mechanisms by which redox-inactive metals induce oxidative stress include binding to thiol (–SH) groups of enzymes and proteins which in turn leads to the depletion of thiol pool, inhibition of antioxidant defense systems, and increased lipid peroxidation (Fig. 5.1).

5.2 Lead

Lead is a bluish-gray metal found naturally in the earth's crust. Because of its versatility, lead has been widely used since civilization in paints and dyes, glazed ceramics, gasoline as an antiknock additive, leaded battery, and lead alloys and even to sweeten wine (ATSDR 2007). Humans are exposed to lead through inhalation of lead bound to dust and chemicals and ingestion of lead-contaminated food and drinking beverages. Once it enters the body, lead circulates in the blood and reaches various target organs. In adults, more than 90 % of the total body burden of lead is

stored in the skeleton (Saltzman et al. 1990). Although lead in the bones may not be readily toxic, it can be released out of the bones and redistributed to the blood during periods of increased bone resorption such as pregnancy or aging (Hu et al. 1998, 2007). A recent National Toxicology Program (NTP) review concluded that there is sufficient evidence that even low-level exposures for a long period are associated with adverse health effects including neurological, cardiovascular, and renal outcomes (NTP 2012).

5.2.1 Lead and Oxidative Stress

Because lead is a redox-inactive metal, it is known to induce oxidative stress through indirect mechanisms including depletion of thiol groups and damage to antioxidant defense systems. Glutathione (GSH) is a tripeptide containing the thiol group on the cysteine residue and plays an important role in the protection of cells against oxidative damage (Dickinson and Forman 2002). Lead shows a high affinity for GSH, which leads to a reduction in GSH levels and activity in the liver, brain, lungs, and eye lens (Neal et al. 1999; Samarghandian et al. 2013; Sandhir et al. 1994; Sandhir and Gill 1995). Glutathione peroxidase (GPx), catalase (CAT), and superoxide dismutase (SOD), which enzymatically scavenge free radicals, are also potential targets for lead (Farmand et al. 2005; Ni et al. 2004). Lipid peroxidation has also been reported as an effect of lead-induced oxidative stress (Lawton and Donaldson 1991; Yiin and Lin 1995). Numerous epidemiologic studies have also shown associations between lead exposure and markers of oxidative stress such as malondialdehyde (MDA) and γ-glutamyltransferase (GGT), even at low-level exposure in the general population. MDA is used as a biomarker of lipid peroxidation as ROS induce MDA by degrading polyunsaturated fatty acids (Del Rio et al. 2005). Dose-dependent associations between lead exposure and MDA were found in lead-exposed workers, urban adolescents, or children living near a landfill (Ahamed et al. 2006; Cabral et al. 2012; Kasperczyk et al. 2013; Moro et al. 2010; Oktem et al. 2004). Serum GGT has been proposed to be an oxidative stress marker given its role in metabolizing extracellular GSH and providing cysteine for intracellular GSH synthesis (Lee et al. 2004). Lee et al. examined adult participants in the third US National Health and Nutrition Examination Survey (NHANES III) and found strong dose-dependent associations of blood lead with serum GGT as well as other serum biomarkers of antioxidant vitamins (carotenoids, vitamin C, and vitamin E) (Lee et al. 2006).

Lead is also known to increase oxidative stress by interacting with heme biosynthesis. Over 99 % of the lead in blood is partitioned predominantly within red blood cells (RBCs), where most lead is bound to δ-aminolevulinic acid synthetase (δ-ALAS) (Bergdahl et al. 1997). Lead can inhibit δ-ALAS activity due to its high affinity for thiol groups, which results in accumulation of δ-ALA. In lead poisoning, ALA undergoes auto-oxidation and generates superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\bullet}OH$) (Bechara 1996), which was also evidenced by the inhibition of antioxidant enzymes (e.g., SOD and CAT) (Monteiro et al. 1986).

5.2.2 *Lead and Ototoxicity*

Hearing loss is a degenerative disease which results from cell death in the cochlea, including degeneration of the organ of Corti, ganglion cell loss, strial atrophy, and basilar membrane stiffness (Liu and Yan 2007). Oxidative stress in mitochondria and reduced blood flow in the cochlea play a pivotal role in the degeneration of the cochlea (Le Prell et al. 2007; Seidman et al. 2004), and lead-induced oxidative stress potentially through mechanisms discussed above may lead to ototoxicity. Other potential mechanisms include neuro-ototoxic effects of lead on the auditory brainstem and cochlea (Bertoni and Sprenkel 1988; Lasky et al. 1995; Yamamura et al. 1989), inhibition of ion flow through calcium channels (Audesirk 1993; Garza et al. 2006), and disruption of plasma membrane calcium pumps, resulting in loss of sensory hair cells and hearing loss (Brini 2009; Schultz et al. 2005; Shull et al. 2003; Spiden et al. 2008).

Tuncel et al. examined ototoxic effects of lead acetate (inorganic lead) and tetraethyl lead (organic lead) using direct ROS-generating systems (e.g., $O_2^{\bullet-}$, H_2O_2, $^{\bullet}OH$) in the cochlea of guinea pigs (Tuncel et al. 2002). By comparing ROS level and measures of cochlear function, e.g., compound action potential (CAP) thresholds and changes in cochlear microphonic (CM) sensitivity, ROS generation has been shown to be an important pathway for lead-induced ototoxicity (Clerici and Yang 1996). Tetraethyl lead-exposed guinea pigs had significantly higher CAP thresholds in mid to high frequencies compared to controls. The lead acetate-exposed group also had higher (poorer) CAP thresholds than controls but less than the tetraethyl lead-exposed group. Pretreatment with α-phenyl-*tert*-butyl-nitrone, a free radical scavenger, significantly diminished tetraethyl lead-induced threshold shifts but not those induced by lead acetate. There were no differences in CM sensitivity between either of the lead groups and controls. These findings suggested that free radicals generated by lead exposure, especially tetraethyl lead, at least partially mediate lead toxicity in the inner ear (Tuncel et al. 2002). Importantly, they also indicate that the primary target for lead toxicity in the inner ear is not the sensory cells (little change in the CM) but the afferent fibers of the auditory nerve (reduced CAP).

Brainstem auditory evoked potential (BAEP, also known as brainstem auditory evoked response or auditory brainstem response [ABR]) provides a measure of nerve conduction velocity from the ears to the brainstem (Bhattacharyya 2013). BAEP waveform components represent the CAP in the distal portion of cranial nerve (wave I), second-order neuron activity beyond the cranial nerve in or near the cochlear nucleus (wave III), and activity in higher auditory structures (wave V). Interpeak latencies (i.e., I–III, III–V, or I–V) reflect changes in peripheral auditory nerve latency from changes in brainstem transmission in the auditory pathway.

In the occupational setting with lead-exposed workers, blood lead levels were associated with longer BAEP wave latencies (Bleecker et al. 2003) and higher auditory hearing thresholds (Chuang et al. 2007; Hwang et al. 2009). In a study conducted with 259 steel plant workers in Taiwan (mean blood lead level = 5.43 μg/dL) (Hwang et al. 2009), the odds ratios (ORs) of hearing loss (defined by threshold >25 dB HL) comparing people with blood lead levels ≥7 μg/dL with those with

blood lead levels <4 μg/dL were 4.49 (3 kHz), 6.26 (4 kHz), 3.06 (6 kHz), and 6.16 (8 kHz). In a study of 30 lead-glazing Andean workers in Ecuador who had the mean blood lead levels of 45 μg/dL (standard deviation (SD) 19.5), there were dose-dependent associations between blood lead levels and delayed BAEP wave latencies (Counter and Buchanan 2002).

Two epidemiologic studies of the association between low-level lead exposure and hearing loss in the general population of adults have been reported (Choi et al. 2012; Park et al. 2010). In a study of 3,698 US adults aged 20–69 years using data from the National Health and Nutrition Examination Survey (NHANES) 1999–2004 (geometric mean blood lead = 1.54 μg/dL), individuals in the highest quintile of blood lead had 18.6 % (95 % confidence interval (CI), 7.4–31.1 %) higher hearing thresholds (pure-tone averages (PTA) at 0.5, 1, 2, and 4 kHz) than did those in the lowest quintile, with a significant trend across quintiles (p for trend < 0.001), after adjustment for sociodemographic and clinical risk factors and exposures to occupational and nonoccupational noise (Choi et al. 2012). Park et al. examined the associations between cumulative lead exposure measured by bone lead levels and hearing thresholds in 448 elderly community-dwelling men in Boston, Massachusetts, USA (Park et al. 2010). Patella bone lead levels were significantly associated with higher (poorer) hearing thresholds at 2–8 kHz, especially at the frequency of 4 kHz, and with higher odds of hearing loss (defined as PTA > 25 dB, OR = 1.48 (95 % CI, 1.14–1.91)), even after adjustment for important risk factors for hearing loss including occupational noise exposure. They also found a significant positive interaction between tibia bone lead and age in the longitudinal trajectory of hearing thresholds over up to 32 years of follow-up, suggesting that cumulative lead exposure may accelerate age-related hearing loss.

Several epidemiologic studies have also reported an association between lead exposure and hearing impairment among children. Two national surveys in the USA (NHANES II and Hispanic Health and Nutrition Examination Survey (HHANES)) examined children and adolescents (6–19 years of age) and found significant positive associations between blood lead and hearing thresholds at four frequencies (0.5, 1, 2, and 4 kHz) even at blood lead levels less than 10 mg/dL (Schwartz and Otto 1987, 1991). In a study conducted in an industrial area in Poland where 155 children aged 4–14 years with the median blood lead levels of 7.2 μg/dL were examined, blood lead levels were significantly associated with higher hearing thresholds at all frequencies from 0.5 to 8 kHz and with prolonged BAEP latency of wave I (Osman et al. 1999).

An interesting hypothesis was recently proposed. Lead poisoning is the primary cause of deafness of Ludwig van Beethoven (Stevens et al. 2013). The authors conducted an extensive review of the musical and medical literature and analyzed the information related to diagnoses for Beethoven's hearing loss. They argued that evidence of the potential etiology proposed before including otosclerosis, autoimmune inflammatory bowel disease, mercury poisoning, and syphilis is lacking. After Beethoven's mother died when he was 17 years old, he began to drink wine, particularly the adulterated or fortified Hungarian wine, and became alcohol dependent. It is well known that at that time lead was added illegally to sweeten wine,

especially inexpensive wine. The authors concluded that *Beethoven's chronic consumption of wine tainted with lead is a better explanation of his hearing loss than other causes*.

5.3 Cadmium

Cadmium is a trace element that has no essential function in humans. Cadmium, naturally found in the earth's crust, is a by-product of the mining of zinc, lead, and copper ores. Cadmium is a serious soil contaminant from industrial waste and phosphate fertilizers, which eventually contaminates vegetables and fruit (ATSDR 2012). This makes most human foodstuffs a primary nonoccupational source of cadmium exposure within the general population (Satarug et al. 2010). Cigarette smoking is not only the major source of cadmium exposure among smokers but also an important cadmium exposure source even among nonsmokers due to secondhand smoke (Tellez-Plaza et al. 2012).

Various organ systems can be affected by cadmium exposure. A notable chronic high-dose effect is itai–itai (ouch–ouch) disease; cadmium poisoning occurred in the 1910s in Toyama Prefecture, Japan, characterized by softening of the bones and kidney failure (Emmerson 1970). Cadmium is a Group I carcinogen as determined by the International Agency for Research on Cancer (IARC) (Straif et al. 2009). Low-level chronic exposure has also been associated with kidney dysfunction, osteoporosis, cardiovascular disease, lower lung function, and diabetes (Satarug et al. 2010).

5.3.1 Cadmium and Oxidative Stress

Three potential mechanisms for cadmium-induced oxidative stress have been proposed (Cuypers et al. 2010): (1) induction of NADPH (nicotinamide adenine dinucleotide phosphate) oxidases, (2) replacement of redox-active metals, and (3) inhibition of antioxidant defense system including cadmium–thiol complexes formation.

NADPH oxidases generate superoxide ($O_2^{\bullet-}$) by transferring electrons derived from intracellular NADPH (Quinn et al. 2006). Superoxide can generally produce hydrogen peroxide (H_2O_2) spontaneously or through SOD. Despite its important function to kill bacteria and fungi, excess production may cause lipid peroxidation and oxidative damage. Cadmium exposure was found to upregulate NADPH oxidase gene expression in mouse kidneys (Thijssen et al. 2007) and hepatoma cell lines (Fotakis et al. 2005), which increases NADPH oxidase activity and in turn increases superoxide production. Another potential pathway is that cadmium replaces iron in various proteins and enzymes and, therefore, increases the free iron concentration (Dorta et al. 2003). Cadmium can mimic iron due to the same valency. Free redox-active metals

like iron produce potent free radicals through the Fenton reaction followed by the Haber–Weiss reaction (Ercal et al. 2001; Jomova and Valko 2011).

Thiols play a critical role in cadmium-induced oxidative stress. Thiols, such as GSH and metallothionein, are known to detoxify cadmium by the formation of cadmium–thiol complexes, which reduces free cadmium and then blocks the aforementioned pathways leading to cadmium-induced oxidative stress (Thevenod 2009; Zalups and Ahmad 2003). Acute cadmium exposure has resulted in decreases in GSH and other antioxidants (e.g., SOD and CAT) and their activities (Casalino et al. 2002; Yalin et al. 2006). By contrast, chronic low-level exposure seems to activate those antioxidant enzymes, which in turn protect against cadmium-induced oxidative damage (Cuypers et al. 2010), a phenomena that mimics the "conditioning" effect of low-level noise, which similarly upregulates antioxidant defenses and decreases vulnerability of the inner ear to subsequent insult (Jacono et al. 1998). However, long-term persistent exposure to cadmium even at low levels may cause the antioxidant defense systems to fail, leading to an oxidative challenge with stress and aging (Ercal et al. 2001).

5.3.2 Cadmium and Ototoxicity

Ozcaglar et al. reported that cadmium can accumulate in ear ossicles and the labyrinth in rats (Ozcaglar et al. 2001). They also found that cadmium exposure at a concentration of 15 ppm $CdCl_2$ caused defective hearing and kidney dysfunctions, but 5 ppm $CdCl_2$ exposure caused only hearing loss without kidney dysfunction, suggesting that hair cells are more susceptible to cadmium than kidney tubule cells. Cadmium exposure increased the mean latency of ABR wave I but did not change wave III and V latencies. This result suggests that the cochlear component of hearing is more sensitive to cadmium toxicity than other, central, parts of the auditory system (Ozcaglar et al. 2001). Kim et al. examined cadmium-induced ototoxicity using auditory cell lines (HEI-OC1) and a mouse model (Kim et al. 2008, 2013). Cadmium exposure induced ROS generation, loss of mitochondrial membrane depolarization, the release of cytochrome c, activation of caspases, apoptosis, production of proinflammatory markers (interleukin (IL)-1β and IL-6), an increase of extracellular signal-regulated kinase activation in HEI-OC1 cells, and an elevation in ABR thresholds in mice. These adverse effects were significantly prevented by treatment with *N*-acetyl-L-cysteine (NAC), an antioxidant precursor to GSH (Dodd et al. 2008), and rosmarinic acid, a caffeic acid ester with antioxidant properties found in medicinal plants (Petersen and Simmonds 2003). They also observed that hair cells and Hensen cells were more responsive than Claudius cells to cadmium-induced apoptosis, which suggests that cadmium exposure may affect the basilar membrane vibration and sensory transduction and block nerve impulses transmitted to the brain via auditory nerves (Kim et al. 2008).

Two epidemiological studies reported the association between cadmium exposure and hearing loss. Shargorodsky et al. examined 875 adolescents aged

12–19 years who participated in the US NHANES 2005–2008 (Shargorodsky et al. 2011). After adjusting for important confounders, adolescents in the highest urinary cadmium quartile had a threefold higher odds of low-frequency hearing loss (defined as PTA at 0.5, 1, and 2 kHz > 15 dB) compared to the lowest quartile (odds ratio (OR) = 3.08 (95 % CI, 1.02–9.25)). Choi and colleagues investigated 3,698 US adults aged 20–69 years using data from the NHANES 1999–2004 (Choi et al. 2012). After adjusting for sociodemographic factors, occupational and nonoccupational noise exposures, and blood lead levels, the highest quintile group had a 74 % higher odds of hearing loss (defined as PTA at 0.5, 1, 2, and 4 kHz > 25 dB) compared to the lowest quintile group (OR = 1.74 (95 % CI, 1.12–2.70), with a significant trend across quintiles (p for trend = 0.01).

5.4 Mercury

Mercury is a transition metal that has several chemical forms. Metallic mercury (HgO) is a shiny, silver-white liquid used in thermometers, dental amalgams, and batteries (ATSDR 1999). Mercury can also form inorganic and organic compounds with salts (e.g., chlorine) or carbon that are typically exposed through ingestion. The most common and the most toxic form is methylmercury which can cross biological membranes, such as the blood–brain barrier and placenta, rendering it an important neurotoxicant (Aschner and Aschner 1990). Methylmercury is bioaccumulated in fish and seafood through the food chain, and thus, methylmercury concentrations are generally higher in large, long-lived fish (e.g., tilefish, shark, swordfish) and lower in small, and short-lived ones (e.g., salmon, pollock, catfish, shrimp) (ATSDR 1999). Metallic or inorganic forms of mercury through dental amalgam or occupational exposure (e.g., industrial use and gold mining) are also toxic to various degrees. Potential adverse effects of mercury exposure include neurological dysfunction, cardiovascular events, renal dysfunction, and dysfunction in endocrine, reproductive, and immune systems (Karagas et al. 2012; Rice et al. 2014). A notorious episode of severe mercury poisoning is Minamata disease, which occurred in the 1950s in Minamata Bay, Japan, where a chemical factory discharged large quantities of a mercury catalyst into the bay (Goldman 2007). Acute exposure to methylmercury caused paresthesia, ataxia, tremor, muscle spasticity, and visual and hearing impairment (Goldman 2007).

5.4.1 Mercury and Oxidative Stress

Mercury can induce oxidative stress through three potential mechanisms (Farina et al. 2011): (1) interaction with thiol and/or selenol (–SeH) groups from endogenous molecules, (2) disruption of mitochondrial electron transfer chain, and (3) an increase in extracellular glutamate levels and subsequent intracellular calcium influx and excitotoxicity.

Numerous studies have shown that both methylmercury and metallic mercury exposure can perturb the activities of proteins/enzymes containing thiol and/or selenol groups, such as GSH, GPx, Ca^{2+}-ATPase, thioredoxin reductase, choline acetyltransferase, and enolase (Farina et al. 2013). Because selenols are more nucleophilic than thiols (Sugiura et al. 1976), mercury compounds have a higher affinity for selenols and hence preferentially bind to selenoproteins such as GPx and thioredoxin reductase (Branco et al. 2012; Farina et al. 2013). Thiol-containing proteins in mitochondria, such as respiratory chain complexes and mitochondrial creatine kinase, are also primary targets of methylmercury (Glaser et al. 2010), which can lead to mitochondrial collapse and mitochondrial oxidative damage (Franco et al. 2007). Methylmercury can also disrupt the complexes II (succinate dehydrogenase) and III (cytochrome bc1 complex)-mediated pathway in the mitochondrial electron transport chain, leading to the elevated H_2O_2 generation (Franco et al. 2007; Mori et al. 2007).

Disruption of extracellular glutamate homeostasis and subsequent calcium influx into neurons has been suggested as mercury-induced oxidative stress and neurotoxicity (Farina et al. 2011, 2013). Mercury compounds (e.g., methylmercury and inorganic mercury) lead to increased extracellular glutamate levels via the inhibition of astrocyte glutamate uptake (Aschner et al. 2000; Brookes and Kristt 1989) and the stimulation of glutamate release from presynaptic terminals (Albrecht and Matyja 1996; Reynolds and Racz 1987). Elevated extracellular glutamate can overactivate *N*-methyl-D-aspartate (NMDA)-type glutamate receptors, which results in calcium influx into postsynaptic neurons (Lafon-Cazal et al. 1993), leading to the activation of neuronal nitric oxide synthase (nNOS) and increased formation of nitric oxide (NO) (Himi et al. 1996; Yamashita et al. 1997), a reactive nitrogen species that can cause harmful oxidative and nitrosative stress responses (Thomas et al. 2008).

5.4.2 Mercury and Ototoxicity

Ototoxic effects of mercury compounds have been reported in animal models. Lin-Shiau and colleagues found that methylmercury and mercuric sulfide (HgS, also known as cinnabar, a naturally occurring HgS compound used as a sedative for more than 2,000 years in Asia (Kang-Yum and Oransky 1992)) were accumulated in the brainstem and caused increased hearing thresholds and prolonged absolute and interwave latencies of ABR in a mouse model (Chuu et al. 2001; Huang et al. 2008). They also observed suppressed Na^+-/K^+-ATPase activity, elevated NO levels, and lipid peroxidation in the brainstem in a dose-dependent manner. These results suggest that mercury-induced oxidative stress due to excess NO formation, lipid peroxidation, and altered Na^+-/K^+-ATPase activity in the brainstem could be an important pathway of mercury-induced ototoxicity. They also found that mercury exposure during the developmental periods (gestation and lactation) induced the mercury ototoxic effects listed above in offspring mice, suggesting that fetuses are vulnerable to mercury ototoxicity (Huang et al. 2011). Chronic low-level exposure to methylmercury (50 μg/kg/day) from birth to 7 years of age in monkeys caused

high-frequency hearing loss at age 14 years, which also suggests the developmental period as a window of susceptibility to mercury ototoxicity (Rice and Gilbert 1992).

In human populations, high exposures to mercury compounds at workplaces or from contaminated community areas have been associated with adverse hearing outcomes. Auditory disturbances and hearing impairment were reported in Minamata disease patients (Harada 1995) and residents living near the Minamata Bay who have consumed fish and shellfish contaminated with low-level methylmercury (Ninomiya et al. 1995).

Different biomarkers reflect different mercury species exposures. Hair mercury mainly indicates methylmercury exposure, blood mercury indicates both methylmercury and elemental mercury exposure, while urinary mercury mainly indicates elemental mercury exposure (CDC 2013).

In a study of 26 mercury workers in Taiwan, Chang et al. reported prolonged wave III (5.73 ms vs. 5.80 ms) and IV (4.15 ms vs. 4.26 ms) latencies in the top one third ($n = 10$, hair mercury level = 53.9 (SD 34.6) μg/g) compared to the bottom one third ($n = 11$, hair mercury level = 4.9 (SD 1.1) μg/g) (Chang et al. 1995). In a study of 39 nonsmoking female dentists (age 40–45 years), there were significant positive associations between the number of amalgam fillings they had performed on patients and hearing thresholds at high frequencies (8, 11.2, 12.5, 14, and 16 kHz) (Rothwell and Boyd 2008). A cross-sectional study of 138 male workers of a fluorescent lamp factory in Egypt reported that individuals with hearing impairment had higher urinary mercury levels (51.4 (SD 19.5) μg/g creatinine) than those without hearing impairment (42.3 (SD 16.6) μg/g creatinine) (Al-Batanony et al. 2013).

People who work at gold mining areas are at high risk of mercury poisoning which can occur through inhalation of elemental mercury vapor during amalgam burning in the gold extraction process and/or ingestion of methylmercury from contaminated fish. Counter and colleagues examined Andean children and adults living in gold mining areas of Ecuador (Counter et al. 1998, 2012; Counter 2003). In children whose ages were between 4 and 14 years (sample sizes ranged from 22 to 31), blood mercury levels ranged from 2 to 89 μg/L with medians of 7–23 μg/L, which are much higher than the median blood mercury level of 0.26 μg/L reported for children in the USA (Schober et al. 2003). There were significant positive correlations between blood mercury levels and the absolute latency of wave V ($r = 0.38$, $p = 0.03$) and the I–V interwave interval ($r = 0.41$, $p = 0.02$) (Counter 2003). They also observed significant positive correlations between blood mercury and hearing thresholds (Counter et al. 1998) and acoustic stapedius reflex thresholds (Counter et al. 2012) in children. In the same studies (Counter et al. 1998, 2012), they also examined adult gold mining workers who had blood mercury levels between 2 and 32 μg/L with a median of 6 μg/L which is also higher than that found in the US general population (geometric mean of 1.03 μg/L) (Park et al. 2013). However, surprisingly, no significant associations between blood mercury and hearing outcomes were found in adults.

Little is known about the association between low-level exposure to mercury and hearing outcomes. Shargorodsky et al. examined 2,535 adolescents who participated in NHANES 2005–2008 but found no association of blood mercury with either low- or high-frequency hearing loss (Shargorodsky et al. 2011).

Given that methylmercury can cross the placenta (Aschner and Aschner 1990), prenatal exposure to methylmercury is of concern. Studies based on a birth cohort in the Faroe Islands ($N = 800$–900) examined the association between cord blood mercury (range, 13.4–41.3 μg/L; geometric mean = 22.9 μg/L) and BAEPs at 40 Hz assessed at 7 years (Grandjean et al. 1997) and 14 years of age (Murata et al. 2004). At age 7, every log-unit increase in cord blood mercury was associated with increased latencies of 0.043 ms ($p = 0.10$) at wave I, 0.053 ms ($p = 0.06$) at wave III, and 0.059 ms ($p = 0.01$) at wave V (Grandjean et al. 1997). At age 14, the corresponding associations were 0.027 ms ($p = 0.09$) at wave I, 0.032 ms ($p = 0.05$) at wave III, and 0.048 ms ($p = 0.01$) at wave V (Murata et al. 2004).

5.5 Conclusions

High exposures to lead, cadmium, and mercury at levels found in the occupational setting have consistently been reported to increase the risk of hearing loss. A body of evidence suggests that ROS formation and oxidative stress induced by these redox-inactive metals play a critical role in the etiology of hearing loss. However, there is insufficient evidence of the effect of low-level exposure to heavy metals conducted in the general population. In addition, few epidemiologic studies have been conducted with a prospective study design, raising concerns related to causal inferences.

Long-term low-level exposure is of most concern as the aging population grows. Lead, cadmium, and mercury are persistent heavy metals and can accumulate in the body. Older adults who are at greatest risk for hearing loss have had greater exposure to ototoxic heavy metals throughout their lives. Current blood levels of heavy metals which reflect only recent exposure may not capture the actual risk due to cumulative exposure. Further studies using biomarkers of cumulative exposure, such as bone lead and urinary cadmium, need to be conducted to better understand the effect of long-term low-level exposure on hearing loss.

Another important consideration is how to prevent ototoxic effects of heavy metal exposures. More efforts to reduce exposure to heavy metals certainly should be recommended. However, this may not be very useful for those who already had high, cumulative exposures through both occupational and environmental exposures, including old adults. An overwhelming body of evidence has demonstrated that higher intakes of fruit and vegetables, i.e., antioxidants, are associated with lower rates of mortality, cancer, cardiovascular disease, and other chronic diseases (Cooper et al. 2012; Hartley et al. 2013; Jung et al. 2013; Koushik et al. 2012). However, little is known about the roles of antioxidant nutrients in mitigating the adverse health effect of heavy metals. Establishing a reliable interaction between antioxidant nutrients and heavy metals would have an important public health implication because dietary nutrient intake recommendation may be an economical and efficient intervention, if there is reliable evidence that can be used to guide medical recommendations. Such research may provide a more direct test for the role of free radicals and oxidative stress as potential biological mechanisms by which heavy metals affect hearing loss.

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Chapter 6
The Role of Nutrition in Healthy Hearing: Human Evidence

Christopher Spankovich

A major function of human epidemiological studies is to identify factors that may exacerbate or mitigate susceptibility to disease and disorders and contribute to interindividual susceptibility. Hearing loss is a multifactorial disorder dependent on biological and environmental determinants. These factors can fall into three primary categories: *modifiable*, *non-modifiable*, *and possibly modifiable determinants*. Table 6.1 provides examples of the determinants that have been proposed for acquired hearing loss (here defined as noise, ototoxic drug, and aging factor related).

The major limitation of epidemiological studies in general is related to study design. The majority of epidemiological data available are cross-sectional in nature; therefore, while it is often possible to identify a clear relationship/association between factors, it is not possible to attribute causal implications to the relationship. Diet represents a *modifiable variable* that may have both direct and indirect implications for hearing health. For example, a healthy diet may provide exogenous antioxidants and precursors for endogenous antioxidants to enhance protection of cochlear structures. In addition, a healthy diet may decrease risk for cardiovascular disease and metabolic disorders and in turn decrease risk for hearing loss.

The animal-based literature demonstrating significant effects of dietary nutrients and phytochemical compounds on susceptibility to various forms of acquired hearing loss is growing exponentially. Animal studies of age-related hearing loss (ARHL), noise-induced hearing loss (NIHL), and ototoxic drug-induced hearing loss (OIHL) are primary targets for nutrient- and phytochemical-based otoprotection strategies. Le Prell and Spankovich (2013) provide a recent review of animal-based findings. However, the major limitation of animal models is they are simply not human. Animals of other species do not have the exact same dietary requirements

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Table 6.1 Determinants of hearing loss

Modifiable	Non-modifiable
Noise and hearing protection use	Age
Diet	Sex
Other lifestyle (smoking, alcohol consumption, exercise)	Race/ethnicity
Other environmental determinants (e.g., chemicals)	Genetics
Possibly modifiable	
Health, disease, medications	
Socioeconomic status/education	

or biochemistry as humans. For example, the majority of mammals endogenously produce vitamin C, humans do not, and we must acquire this vital nutrient exogenously (Baker 2008). Though this does not diminish the importance animal research has contributed to understanding of nutritional biochemistry and in understanding auditory physiology and pathology, it remains a potentially confounding factor that requires consideration. In contrast, the data available in human populations is much more limited.

One of the earliest attempts to examine influence of diet on auditory function was performed by a surgeon named Samuel Rosen over 50 years ago. Rosen conducted ecological studies comparing the hearing of people living in different countries. His seminal report (Rosen et al. 1962) was based on data collected in the Mabaan tribe in Sudan. The tribe was described as being relatively noise-free with frugal diets and nearly nonexistent evidence of cardiovascular disease even in the oldest members. Audiometry demonstrated essentially normal hearing sensitivity through the eighth decade of life. However, Rosen and colleagues did not measure dietary outcomes; rather, they were focused on cardiovascular health and implications for hearing.

Upon returning from Sudan, Rosen collaborated with an ongoing study examining saturated fat intake and risk for coronary heart disease in Finland (Turpeinen et al. 1968). In the study two mental hospitals with reported comparable patient populations were assigned to dietary manipulation. In the experimental hospital, the diet was changed so that a large part of the normal saturated fat of the Finnish diet was replaced by soybean oil, whereas the control hospital was kept on the standard higher saturated fat Finnish diet. The initial findings demonstrated that lower saturated fat diet reduced serum cholesterol soon after diet change and reduced electrocardiographic evidence of coronary heart disease within a 3-year period (Turpeinen et al. 1968).

Five years after the start of the dietary manipulation, audiometry was performed on the patients. They found in an age-matched comparison that the patients in the experimental diet hospital had significantly better hearing sensitivity compared to the control diet hospital patients (Rosen and Olin 1965). Next, the diets of the two hospitals were reversed and again followed for a 3-year period. Interestingly, a reversal in the cardiovascular effects began to show. In addition, audiometry was reassessed and showed that patients in the previous experimental hospital (low saturated fat diet), now on the higher saturated fat diet, began to show a trend toward poorer hearing, while the opposite effect was observed in the previous control diet

hospital patients, now on the lower saturated fat diet (Rosen et al. 1970). This series of studies represented some of the first evidence of not only the influence of diet on hearing but also diet on cardiovascular health in humans and cardiovascular health on hearing.

6.1 Epidemiology of Diet and Hearing: 1970s–1980s

A study in 1975 from Edinburgh, Scotland, provided a blip in the radar screen for research on diet and hearing (Lonergan et al. 1975). In this study a dietary assessment was performed in approximately 500 participants randomly selected from a defined area in Edinburgh. Clinical measures, including pure-tone audiometry, were performed. The results indicated that men with deficient diets had higher prevalence of hearing loss. However, the specific nutrients associated with hearing loss were not detailed. In addition, Gosselin and Yanick (1976) reported improved hearing and relief from tinnitus following dietary intervention in patients with metabolic dysfunction; specific dietary recommendations were not provided.

The1970s and 1980s had very few studies in human populations examining the relationship between diet and hearing. However, there was a growing literature of risk factors for hearing loss including cardiovascular and metabolic disorders that are highly related to diet (e.g., hyperlipoproteinemia and diabetes) and investigations into low-salt diets as a conservative management option in Meniere's disease (Booth 1977; Axelsson et al. 1979; Boles et al. 1975, Shea and Konishi 1969).

6.2 Epidemiology of Diet and Hearing: 1990s–2000s

Based on evidence from animal findings, investigators began examining associations of specific nutrients and hearing. For example, in 1989, zinc deficiency was suggested as a contributing factor to hearing loss, based largely on high levels of zinc observed in normal cochlear tissue. Zinc supplementation was reported to improve tinnitus and hearing in patients with marginal zinc deficiency (Shambaugh 1989). However, the majority of these studies have focused on a select group of nutrients, most commonly lipids (e.g., cholesterol and triglycerides), B vitamins, or nutrients that have antioxidant properties as mediators of hearing loss.

6.2.1 Lipids: Serum Measures and Dietary Intake

Numerous studies have explored serum lipid levels or the presence/absence of hyperlipidemia as related to hearing loss (Jones and Davis 2000, 2001; Suzuki et al. 2000; Lee et al. 1998; Evans et al. 2006; Dullemeijer et al. 2010; Simpson et al. 2013). However, findings have been inconsistent. For example, Jones and Davis (2000)

found in a retrospective study that subjects with hyperlipidemia (as measured by fasting total cholesterol) had better hearing thresholds. On the other hand, Evans et al. (2006) demonstrated that subjects with dyslipidemia (i.e., abnormal lipid profile) as indicated by elevated triglycerides had elevated hearing thresholds compared to matched controls.

The variability may be explained by the blood serum measure used to define lipid status. Cholesterol is a sterol, a vital component of our cells, and the basic building block of hormones. Triglycerides are esters derived from glycerol and three fatty acids, and their primary function is energy supply. Excess calories are repackaged and stored as triglycerides. Cholesterol and triglycerides, both lacking solubility in the blood, are chaperoned in the bloodstream by proteins, thus, as lipoproteins, i.e., lipid + protein. There are several types of lipoproteins based on the order of density from chylomicrons up to high-density lipoproteins (HDL). The lower-density variants have a larger lipid component. The cholesterol component is essentially the same in the different low- to high-density lipoproteins. The density of the lipoprotein has been associated with its relative effect on cardiovascular health, where lower-density lipoproteins (LDL) are associated with poorer cardiovascular outcomes. It is not the cholesterol that represents the risk factor; rather it is the density (molecular weight and saturation) of the associated fatty acids. Triglycerides are a major component of very low-density lipoproteins (VLDL) and when elevated can be indicative of a poor diet and risk for cardiovascular health problems. Cholesterol is usually measured as total cholesterol and two subcomponents: HDL and LDL cholesterol. However, the cholesterol as previously described is essentially the same for these two subcomponents; what differs is the ratio of protein and triglycerides. Therefore, elevated triglycerides (related to higher amount of stored fat and higher VLDL) may be associated with poorer hearing (as in Evans et al. 2006), but total cholesterol may not (Jones and Davis 2000), depending on what constitutes the majority of the lipoprotein transporting that cholesterol (LDL vs. HDL). This may explain why total cholesterol may lack sensitivity, as it comprises both HDL and LDL sources.

Suzuki et al. (2000) performed a cross-sectional study of serum total cholesterol including comparison of LDL and HDL. They demonstrated that total cholesterol and total triglycerides had no relationship to hearing thresholds. However, when subjects with higher HDL were compared to those with lower LDL, a significant relationship was found, where higher HDL was associated with better hearing thresholds and lower LDL with poorer hearing thresholds. In a longitudinal study, Dullemeijer et al. (2010) found that persons with highest levels of serum n-3 polyunsaturated fatty acids had better lower-frequency hearing thresholds. Two studies out of the Medical University of South Carolina (MUSC) found a significant relationship between hearing and ratio of LDL/HDL in a cross-sectional study (Lee et al. 1998), but a follow-up longitudinal study found no significant relationship (Simpson et al. 2013).

A variety of factors can influence serum lipid measures including but not limited to how measured, patient posture, stress, medications, and etc. These factors and recognized acute changes in blood serum lipid measures limit their application for

determining the relationship and risk of hearing loss. The application of serum measures of lipids and relationship to hearing loss remains unclear.

Gopinath et al. (2010a, b, 2011a, b) and Spankovich et al. (2011), studies from the Blue Mountains Hearing Study (BMHS), explored the relationship between dietary lipid intake and auditory function. The BMHS is a population-based longitudinal survey of ARHL in the Blue Mountains of Sydney, Australia, conducted through the years 1997–2004. During 1997–1999, over 3,000 participants 49 years or older were examined; surviving baseline participants were invited to participate in the 5-year follow-up examination between 2000 and 2004; 75 % of the original sample participated in the 5-year follow-up. [The dietary intake of participants was measured via food frequency questionnaires (FFQ). An FFQ is a self-reported inventory of the usual participant consumption of different food items over a length of time (in the case of BMHS over the past year) including portion sizes and frequency of consumption. The FFQ data is entered into a database to estimate the nutrient composition of the reported food intake. The FFQ used in the BMHS studies was validated using weighed food records (Smith et al. 1998).] Audiometric data collected included transient-evoked otoacoustic emissions (TEOAEs), pure-tone threshold sensitivity, immittance measures, and hearing handicap inventory. Information on potential confounders was obtained via a comprehensive medical history. In addition other health data were collected including an evaluation of vision, quality of life, cognitive function, and blood labs (e.g., fasting blood glucose, cholesterol, kidney function, homocysteine, and various serum levels of vitamins).

One of the earliest publications from the BMHS group on diet and hearing was the (Gopinath et al. 2010a) cross-sectional and prospective analysis of dietary intake of polyunsaturated fats and hearing loss. They found that long-chained omega-3 fatty acids were significantly associated with better hearing thresholds (cross-sectional) and reduced 5-year incidence of hearing loss (prospective). In addition, higher intake of fish was associated with reduced risk and progression of hearing loss.

Gopinath et al. (2011a, b) in a second cross-sectional and prospective analysis found that higher dietary intake of cholesterol was associated with increased odds of hearing loss. Conversely, serum lipid levels were not associated with odds of hearing loss. In addition, dietary intake of monounsaturated fats was associated with reduced risk of progression of hearing loss over a 5-year period. However, neither total fat intake nor serum lipid levels were associated with 5-year incidence of hearing loss.

Finally, Spankovich et al. (2011) showed that higher total fat and cholesterol intake were associated with poorer TEOAEs. In addition, higher cholesterol intake was associated with poorer high-frequency (3,000, 4,000, 6,000, 8,000 Hz) and low-frequency (500, 1,000, 2,000 Hz) pure-tone averages (PTAs). No further assessments of lipid components or serum measures were analyzed.

In summary, serum levels of lipids have limitations as makers of hearing health and health in general. The overall findings do support a relationship between dietary intake of lipids and hearing (e.g., higher omega-3 fatty acid intake associated with better hearing). However, the effect size may be small.

6.2.2 B Vitamins, Antioxidants, and More

In addition to lipids, antioxidants and other macro- and micronutrients have been a major area of investigation. For example, interest in vitamin B12 and folate (B9) with hearing was based in the common inadequacies of these two nutrients in the elderly population, as well as their respective roles in cellular metabolism, neural integrity, and vascular function.

Increased prevalence of hearing loss was found in older women with low serum levels of vitamin B12 and folate (Houston et al. 1999). On the contrary, another study showed no relationship between serum B12 and folate in elderly subjects (Berner et al. 2000). Gok et al. (2004) found that young adult subjects with evidence of NIHL had higher serum levels of homocysteine and reduced serum levels of B12 and folic acid compared to matched controls. In addition, a significant relationship between ARHL and serum folate was also reported by Lasisi et al. (2010). Gopinath et al. (2010b) examined serum levels of folate, B12, and homocysteine with hearing loss. Higher levels of folate were associated with better hearing and higher levels of homocysteine with poorer hearing prevalence, but no relationship was found prospectively over a 5-year period. Also, no significant relationship was found for B12 serum levels and hearing sensitivity.

Shargorodsky et al. (2010) performed a prospective analysis ($n > 26{,}000$) of vitamin intake and risk of hearing loss in men. The data were collected as part of the Health Professionals Follow-up Study over a period from 1986 to 2004. Based on reported dietary intake and reported diagnosed hearing loss, they found no relationship between intake of vitamins C and E, beta-carotene, and hearing loss. However, dietary folate intake was associated with reduced risk of hearing loss. Dietary B12 was also associated with reduced risk of hearing loss, but only in men with higher alcohol intake. Unfortunately, this study had at least one major limitation; no objective auditory function data was available. Hearing status was based on participant-reported diagnosed hearing loss.

Spankovich et al. (2011) examined 25 total dietary nutrients and relationship to TEOAEs and pure-tone threshold hearing sensitivity (high- and low-frequency PTA). The motivation of the analysis was to determine if associations found in animal-based literature were present in a human population and to identify novel relationships that may inform future directions for otoprotection strategies. All major macro- and micronutrients available in the database were considered including caloric intake, total carbohydrates, protein, total fat, total cholesterol, carotenoids, vitamin A, vitamin C, vitamin E, B vitamins, and trace minerals. Both dietary- and supplement-based sources were included. Prior to adjustment for confounding variables, 19 nutrients showed significant correlations to at least one of three auditory outcomes. After adjusting for significant covariates (age, sex, noise exposure), the findings revealed that better pure-tone threshold sensitivity was associated with higher lycopene, vitamin E, vitamin C, riboflavin (B2), and magnesium intake. Higher intake of lipids, as described above, was associated with poorer auditory outcomes. The major shortcoming of the study was the limitation to

cross-sectional analysis. As a follow-up, Gopinath et al. (2011a, b) examined intake of dietary antioxidants prospectively in the same population. The findings showed that dietary intake of antioxidants was not associated with the 5-year incidence of hearing loss. The lack of significant findings in prospective analysis was attributed to the relatively short time between testings.

Choi et al. (2014) studied the relationship between antioxidant and magnesium intake and hearing in 2,592 participants using the National Health and Nutrition Examination Study (NHANES) database. The NHANES is an ongoing cross-sectional survey of the civilian noninstitutionalized population of the United States. Every 2 years, approximately 10,000 individuals are selected at random within specific demographic distributions so as to be representatives of the US population. The results demonstrated a significant association between higher intake of antioxidants (daily beta-carotene and vitamins C and E) and magnesium with better hearing thresholds.

Gopinath et al. (2010a, b) evaluated the relationship between carbohydrate intake and hearing loss. The results indicated that higher glycemic load (GL) foods (i.e., high carbohydrate and efficiency in raising blood glucose levels) were associated with higher incidence of hearing loss, and higher fiber intake may reduce this risk.

In summary, there are inconsistent findings when examining single-nutrient relationships with hearing. Study design is likely a critical factor (e.g., cross-sectional vs. longitudinal, auditory function measures vs. reported hearing loss). The literature overall suggests there is a relationship between higher intake of nutrients with antioxidant properties and B vitamins for better auditory function. However, longitudinal studies with appropriate controls and measures of auditory function are needed to further understand this relationship.

6.3 Supplements as Otoprotectants: Case–Control Studies

The discussion so far has focused on epidemiological studies of diet-hearing relationships. Numerous clinical studies have also examined the efficacy of macro- and micronutrients in mediating susceptibility to acquired forms of hearing loss in humans by manipulating diet or via supplementation. The first of these types of studies was discussed in the introduction (Rosen and Olin 1965; Rosen et al. 1970).

6.3.1 Age

Somewhat similar to Rosen and Olin (1965) and Rosen et al. (1970), a low-cholesterol diet and anti-lipid therapy were administered to hyperlipidemic patients with tinnitus and hearing loss. The findings indicated reduced subjective tinnitus severity and improved high-frequency thresholds over a 2-year period (Sutbas et al. 2007).

Durga et al. (2007) followed 728 men on folic acid supplementation (800 μg daily) over a 3-year period to assess its influence on hearing. The study was performed in the Netherlands where at that time folic acid fortification of foods was prohibited and baseline folate levels in participants were about half of those found in the US population. The results showed a decreased progression of hearing loss in speech frequencies in participants on the supplement compared to controls. A third case–control study assessed the efficacy of B12 treatment on tinnitus and hearing in 100 patients with B12 deficiency. The treatment showed no effect in ameliorating tinnitus or effect on hearing compared to controls (Berkiten et al. 2013). Takumida and Anniko (2009) assessed the effects of supplementation with rebamipide (300 mg/day), alpha-lipoic acid (60 mg/day), and vitamin C (600 mg/day) on hearing over an 8-week period. The results showed significant improvement in hearing thresholds after treatment primarily limited to low frequencies. The study however had many design limitations including lack of a placebo-control group.

6.3.2 Ototoxic Drugs

Weijl et al. (1998) showed that cisplatin-combination chemotherapy resulted in reduced plasma concentrations of vitamins C and E, uric acid, and ceruloplasmin, despite no significant change in diet. The results provided evidence of reduced antioxidant levels during treatment with cisplatin. In a follow-up study, Weijl and colleagues performed a randomized, double-blind, placebo-controlled study of antioxidant supplementation for cisplatin-induced ototoxicity (Weijl et al. 2004). Participants were supplemented with vitamins C and E and selenium and compared to controls on placebo. No significant difference was found between the supplemented group and placebo in regard to hearing thresholds. However, participants with highest plasma concentrations of the three antioxidants (regardless if on supplement or not) had significantly less loss of higher-frequency hearing. Unfortunately, dietary intake of the participants was not monitored or considered in the analysis. Recent evidence suggests potential limitations of antioxidant supplementation for patients with some forms of cancer; Sayin et al. (2014) found that mice with lung tumors supplemented with antioxidants were seen with an accelerated growth in tumor size and increased mortality.

6.3.3 Sudden Sensorineural Hearing Loss

Based on the evidence of the Weijl et al. (1998) findings, Joachims et al. (2003) performed a prospective, double-blind study of vitamin E in treatment of idiopathic sudden sensorineural hearing loss (ISSNHL). The patients were all treated with steroids, magnesium, and carbogen inhalation. The experimental group received the additional vitamin E (d-α-tocopherol, 400 mg twice daily). The group receiving

vitamin E showed greatest success with nearly 80 % showing at least 75 % recovery of thresholds compared to only 45 % of the control treatment. Similar benefits were described by Hatano et al. (2008). In this study a group of patients received vitamin E (tocopherol nicotinate, 600 mg/day) and vitamin C (1,200 mg/day) in addition to control treatment with steroids. The patients treated with the antioxidant regimen showed an average of 30 dB recovery in thresholds compared to the control's 18 dB recovery.

6.3.4 Noise-Induced Hearing Loss

The application of micronutrient supplement strategies has also been applied to NIHL. Supraphysiological levels of B12 injections (cyanocobalamin, 1 mg/day for 7 days, 5 mg on eighth day) were shown to protect against temporary threshold shift (TTS) in 20 young adult subjects (Quaranta et al. 2004). Two double-blind placebo-controlled studies have reported that Mg can reduce NIHL in humans (Joachims et al. 1993; Attias et al. 1994, 2004). However, it does not appear that individual variation in dietary Mg, in the absence of high-level supplements, is adequate to confer protection against NIHL. In contrast to positive outcomes with higher-level supplements, Walden et al. (2000) reported that plasma Mg was not reliably correlated with NIHL measured in male US Army soldiers with long-term (8–18 years) exposure to high-level weapon noise in a single combat unit.

Le Prell et al. (2011a) based on a series of animal studies (Le Prell et al. 2007, 2011b) examined the combination of beta-carotene, vitamins C and E, and magnesium (ACEMg) for protection from NIHL in Swedish military personnel exposed to gunfire. Unfortunately, the noise exposure among the military personnel did not result in any reliable changes in auditory function. No comparison could be made in regard to potential protection. Clinical trials with ACEMg with a controlled TTS experiment using music players to induce TTS (Le Prell et al. 2012) are concluding, and results should be available soon (NCT: NCT00808470). Other clinical trials using compounds found in trace levels in the diet such as *N*-acetylcysteine (NAC) and D-methionine (D-MET) are also being evaluated.

6.4 Dietary Pattern

So far the studies that have been reviewed have focused on single nutrients or a small group of nutrients with a common function (e.g., antioxidant properties). They have been based on either case–control interventions or epidemiological studies relying primarily on single-nutrient analysis. A single-nutrient analysis involves examining the statistical relationship between a single nutrient and the desired outcome while adjusting for confounding variables. Although this type of analysis has been quite valuable, it may be limited by the complex biochemical and statistical

interactions among nutrients, in addition to the effect of a single nutrient that may be too small to detect, and the increased odds of finding a significant relationship by chance if you are performing 25 comparisons to determine relationships for 25 separate nutrients (Hu 2002).

Another approach to nutrition-disease epidemiology is dietary pattern analysis. Dietary patterns consider types of diets either based on food types (e.g., vegetarian), statistical patterns, or on indices of dietary quality. Previous studies in Japan examined food types as related to susceptibility to ISSNHL. Nakashima et al. (1997) found that subjects who ate more fresh vegetables were at a decreased risk of ISSNHL. The same group later compared diet patterns (Japanese vs. Western diets) and found increased risk for ISSNHL in participants who reported frequent intake of Western foods (Nakamura et al. 2001).

An example of a dietary index is the healthy eating index (HEI). The HEI is a measure of how well a diet conforms to the recommended dietary guidelines of the US Department of Agriculture (USDA). The overall HEI has a total possible score ranging from 0 to 100, with 100 being the "healthy" maximum score. The score is a sum of 10 components each worth 10 points. Components 1–5 measure the degree to which the person's diet conforms to USDA serving recommendations for grains, vegetables, fruits, milk, and meat. Components 6–9 measure total fat as a percentage of total food energy intake, saturated fat as a percentage of total food energy intake, total cholesterol intake, and total sodium intake. Finally, component 10 measures the variety in a person's diet. Components 1–5 are given the maximum score of 10 if the minimal recommended servings for each component are met or exceeded, while components 6–9 are given a maximum score of 10 if intake is less than a certain percentage of their intake or level, while component 10 is given a score of 10 if exceeded at least 16 types of foods in a 24-h period (USDA 1995).

Spankovich and Le Prell (2013) performed the first study of dietary pattern (i.e., HEI) and relationship to hearing in the United States using the NHANES database. Audiometric data were available for 3,853 participants. After excluding participants with missing data, evidence of conductive pathology, and outliers, a final sample size of 2,366 participants were included in the analysis. The analysis examined the relationship between the HEI and pure-tone threshold sensitivity for high (3,000, 4,000, 6,000, 8,000 Hz)- and low-frequency (500, 1,000, 2,000 Hz) PTA.

The results showed a significant relationship between overall HEI score and high-frequency PTA when adjusting for age, sex, race/ethnicity, noise exposure, and education, where higher HEI was associated with better hearing. However, no significant relationship was seen for low-frequency PTA. The relationship was seen across age groups and in both males and females. The subscales showing significant findings included fruit intake, vegetable intake, dairy intake, and variety, where higher scores on each were associated with better high-frequency PTA. Interestingly, a study out of France found that meat intake was associated with better hearing in women and higher intake of seafood was associated with better hearing in men (Péneau et al. 2013).

In a second analysis of the NHANES database, Spankovich and Le Prell (2014) examined the relationship between HEI, PTA, and reported noise exposure.

The results showed a statistically significant interaction between HEI and reported noise exposure with respect to high-frequency threshold sensitivity in participants, where greater reported noise exposure and poorer diet were associated with poorer hearing.

6.5 Summary, Clinical Implications, and Future Directions

The literature overall suggests an influence of diet on auditory function in animals and humans. The relative effect size and specific nutrients that may be involved are less well understood. In general nutrients with functions related to neural integrity, cardiovascular health, metabolic health, and antioxidant properties seem to be associated with better hearing outcomes.

Identification of risk factors that may mediate risk for acquired hearing loss is critical to improving hearing conservation and the public health. There is a large literature of epidemiological studies exploring associations and identifying risk factors for hearing loss including age, sex, noise, race/ethnicity, skin pigmentation, socioeconomic status, medications, cardiovascular health, metabolic health (i.e., diabetes), smoking, secondhand smoke, etc. However, there is a fairly limited study of the relationship between diet and hearing in humans. Understanding the relationship between diet and hearing is also important for understanding other risk factors. For example, smoking and secondhand smoke exposure have been implicated in hearing loss susceptibility (Cruickshanks et al. 1998; Fabry et al. 2011; Agrawal et al. 2009; Gopinath et al. 2010a, b). However, smoking is also associated with poorer HEI (Guenther et al. 2008). In (Spankovich and Le Prell 2014), smoking significantly contributed to the variance for hearing loss when HEI was excluded, but with HEI in the model, smoking was no longer a statistically significant variable.

Diet is an obvious factor for health in general. The benefits of diet have been well known since the ancient Greeks. For example, higher intake of fruits and vegetables is associated with a lower risk of cardiovascular disease and death across numerous studies, while higher intake of saturated fats, sodium, and processed foods has been associated with increased risk of chronic disease (see Liu 2013 for review).

Yet, the role of dietary supplements in prevention of chronic disease/disorders is less clear. Meta-analysis of antioxidant supplements in prevention of chronic disease shows no protective benefit and possible harmful consequences in well-nourished populations (Bjelakovic et al. 2014). For example, the Iowa Women's Health Study showed that use of multivitamins significantly increased risk of all-cause mortality in women (Mursu 2011). Also, the selenium and vitamin E cancer prevention trial (SELECT) study showed that men on maintained supplements of selenium (200 μg/day from L-selenomethionine) and vitamin E (400 IU/day of rac-alpha-tocopheryl acetate) increased risk for prostate cancer (Klein et al. 2011).

Protection conferred by supplements may be limited in populations that practice healthier lifestyles. In addition, supplements are not likely to replace or ameliorate

an unhealthy lifestyle. However, if an individual or group is deficient in a nutrient, supplementation may be of benefit. In developed countries, true dietary deficiencies in key nutrients are less common, much in part due to accessibility, fortification, and supplements. The larger problem is excessive intake of unhealthy foods and reduced intake of healthier foods. Indeed, no studies have shown that antioxidants derived via diet have negative consequences (NIH 2006).

Consider ARHL in animal models. Studies have demonstrated an imbalance of redox status in the cochlea during aging (Staecker et al. 2001; McFadden et al. 2001; Jiang et al. 2007). However, there have been contradictory findings with antioxidant supplementation and hearing in aging animal studies, some studies showing protection (Le and Keithley 2007; Heman-Ackah et al. 2010) and others none (Davis et al. 2007, Sha et al. 2012). Despite the contradictions in ARHL studies, comparable studies of otoprotection strategies for noise and ototoxic drugs show consistent protection mediated by antioxidant agents.

A possible explanation for this seeming discrepancy may be related to the standard diet (i.e., chow) of the control animals. The standard chow in these studies is formulated to meet the nutrient profile recommendations for the species of animal included in the study. In other words, the diet is meeting all the recommended daily requirements of that animal. However, almost no discussion in any of these studies is given to the nutrient profile of the standard diet. This raises the question, how much additional protection is mediated by a diet supplemented with x, y, or z when the diet of that animals is already meeting 100 % of the recommended required intake. It may be possible in the aging studies that the animals are already on adequate diets, and therefore, little additional benefit is conferred by supplementation. [*The effect of lab chow has recently made headlines with diametric effects seen in primate studies of caloric restriction and aging in the Wisconsin and National Institute of Health studies* (*Colman et al.* 2009; *Mattison et al.* 2012).] Conversely, in challenge experiments, such as noise exposure or application of an ototoxic drug, supplements are effective perhaps due to enhanced efficacy to deal with more acute changes that task defense systems.

Humans unlike animals do not eat a standard lab chow that meets daily requirements. For example, the average adult in the United States meets 58 % of the recommended requirements of the USDA, overconsuming foods rich in saturated fat, added sugar, and sodium, while under-consuming fruits and vegetables (USDA 2010). While this may not constitute deficiency in a specific nutrient, it exemplifies the lack of balance in the average US diet and shortcomings of animal models applying supplements to protect against pathology in animals on diets that fulfill daily requirements. It is worth noting that the dietary nutrients that are most commonly inadequate in the US population include vitamins B6, B12, C, and D, iron, and magnesium (CDC 2012).

There is accumulating evidence that a healthier lifestyle including diet may alter susceptibility to acquired hearing loss. More long-term longitudinal studies are needed to determine if causal relationship exists. The use of supplements in otoprotection strategies is encouraging for some acute applications such as noise exposure, ototoxic drugs, and sudden hearing loss. However, the long-term use of supplements

in regard to protection from hearing loss is unknown. Results from the application of supplements for the prevention of other chronic disease/disorders are not encouraging. The optimal source of nutrients remains with our diets, not from supplements that are most commonly synthetic, biochemically unbalanced, and lack the full array of isoforms and phytochemicals found in our foods. Hippocrates said over 2,000 years ago, "Our food should be our medicine. Our medicine should be our food." This remains true today as it did then.

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Part IV
Oxidative Stress and Noise-Induced Hearing Loss

Chapter 7
Basic Mechanisms Underlying Noise-Induced Hearing Loss

Richard A. Altschuler and David Dolan

7.1 Noise-Induced Temporary Threshold Shift

Noise-induced temporary threshold shift (TTS) can occur right after or even during the noise exposure and can resolve almost immediately or take weeks to resolve. By definition, with a "TTS" the thresholds will eventually return to normal levels. The most common metric for assessment in people is a behavioral threshold—the subject indicates when they hear sound, and threshold is defined as the lowest intensity they can consistently report hearing a sound, as tested at different frequencies. The auditory brain stem response (ABR) can also be measured at different frequencies in people, but except in the case of infants, this has very rarely served as a primary outcome in human studies on NIHL. In contrast, in animal subjects, the ABR is the most commonly applied metric (Figs. 7.1 and 7.2), with behavioral assessments (i.e., a trained animal responding when it detects sound) conducted in some studies but being much less common. The measurement of the responsiveness of outer hair cells (OHCs) by otoacoustic emissions (Kemp 2002 for review) can also provide a sensitive objective measure, but the metric only reflects OHC integrity. Otoacoustic emissions are not sensitive to neural damage. Physiological auditory responses from central auditory regions, such as the inferior colliculus, can also be used; or the level of sound needed to generate an acoustic startle reflex can be tested.

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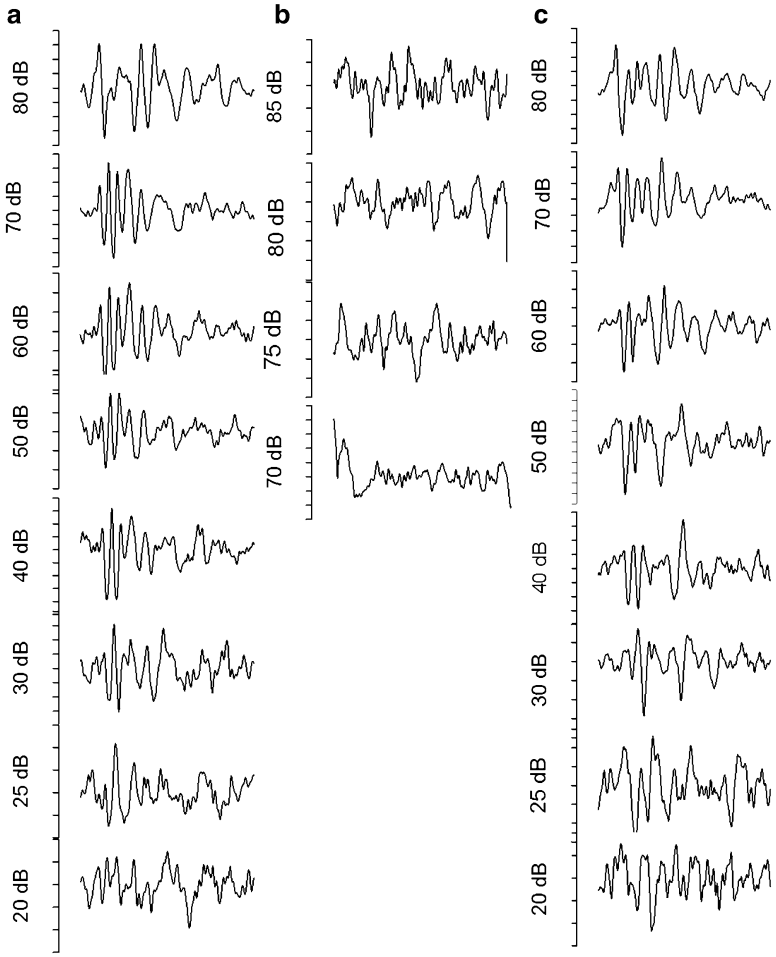

Fig. 7.1 Changes in auditory brain stem response (ABR) following a 106 dB, 2–20 kHz noise for 2 h in a mouse that had only a temporary threshold shift (TTS). The preexposure ABR (**a**) and 3 h (**b**) and 2 weeks (**c**) postexposure are shown. The animal exhibited a large TTS at 3 h (**b**) that recovered by 2 weeks (**c**)

In animals, the functional metric or metrics are often paired with the histopathology of the cochlea. One common assessment of otopathology is a "cytocochleogram" using surface preparations of the cochlear spiral to map the loss or presence of sensory cells (inner hair cells (IHCs) and/or OHCs) along the turns of the cochlea spiral. Figure 7.3 shows a cytocochleogram demonstrating the loss of OHCs following a noise exposure causing noise-induced permanent threshold shift (PTS). Sections through the cochlea can also be used, which can also give information about other cochlear elements. With TTS, one would not expect to see a significant loss of hair cells in a cytocochleogram or sections since the threshold shift generated by such loss would be permanent as hair cells cannot naturally be regenerated or replaced in mammals.

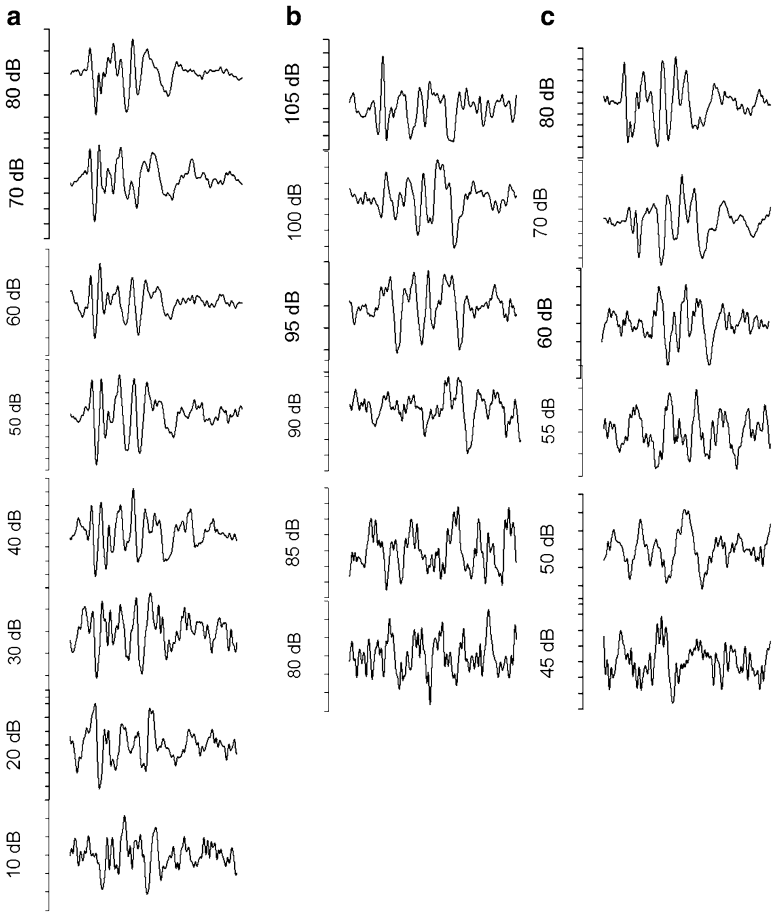

Fig. 7.2 Changes in auditory brain stem response (ABR) following a 106 dB, 2–20 kHz noise for 2 h in a mouse. While receiving the same noise exposure as the mouse in Fig. 7.2, this mouse showed a permanent threshold shift (PTS). The preexposure ABR (**a**), 3 h postexposure (**b**), and 2 weeks (**c**) postexposure are shown. The animal exhibited a large temporary threshold shift (TTS) at 3 h and (**b**) had a remaining PTS at 3 weeks following the exposure (**c**)

A wide variety of noises can cause a TTS, but generally a noise that induces a TTS will be mild to moderate in intensity and duration. Increasing either the intensity or duration of the sound can move a TTS-inducing noise into a PTS-inducing noise. Designing a "TTS" noise exposure involves finding the proper balance of intensity and duration to induce reversible changes, and one can increase either intensity or duration, and decrease the other, and still stay at a TTS-inducing level. Other characteristics, such as a broadband versus narrowband frequency exposure, and variation in the dynamic range or the "impulsive" nature of the sound exposure, will also influence whether a TTS or a PTS occurs. The spectral content of a sound can also influence the cochlear location of the TTS or PTS. Broadband noise will cause broad loss, while an intense pure tone will cause loss at ~1/2 octave above the

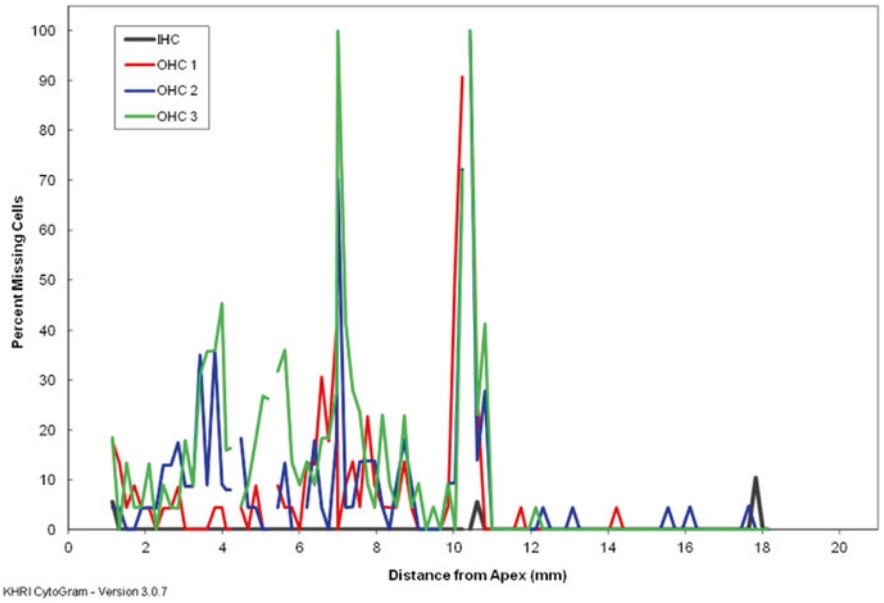

Fig. 7.3 A cytocochleogram from a Hartley guinea pig that received 122 dB, 4 kHz octave band noise for 5 h. Outer hair cell loss in the mid cochlea is associated with a permanent threshold shift

pure tone frequency. There can often be both a TTS and a PTS component following a noise. By definition the TTS component is that which returns to normal levels, and the PTS component is what remains elevated. The mixed changes observed in the early post-noise period are in this case termed a compound threshold shift (Fig. 7.4).

7.1.1 Causes of TTS: General

While TTS is most often considered to reflect some underlying dysfunction, it can also be the result of an intrinsic protective mechanism, muting the effects of an intense sound that could otherwise cause greater damage and PTS. One such reflex targets the middle ear, using interneuron connections in the pons from auditory nuclei to both the motor nucleus of V and to the facial motor nucleus, with branches of the trigeminal and facial nerve then inducing contraction of the tensor tympani and the stapedius middle ear muscles, respectively (Borg and Counter 1989, for review). This causes a decrease in middle ear function for as long as the muscles are contracted, producing a "conduction deafness," i.e., a TTS of about 20 dB. There are also medial efferent reflexes that can influence OHC motility and its amplification effect on the basilar membrane. This is a sound-evoked feedback system to the OHCs that reduces their amplification (Murugasu and Russell 1996; Russell and

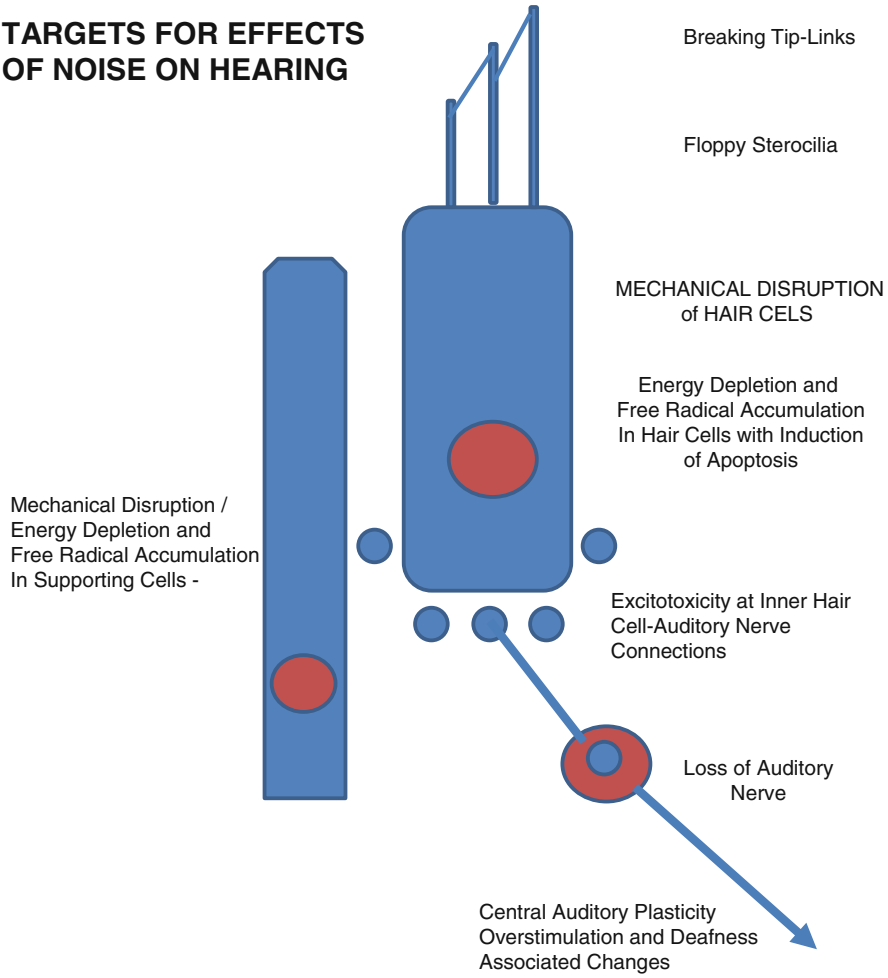

Fig. 7.4 Diagram showing some of the targets for the effects on noise that can cause hearing loss. From top to bottom, these are the tip-links of the inner or outer hair cell (inner hair cell is shown in schematic); hair cell stereocilia that can lose rigidity and become floppy; mechanical disruption, energy depletion, and free radical accumulation in hair cells that can induce apoptosis; mechanical disruption, energy depletion, and free radical accumulation in supporting cells that can disrupt their functions; excitotoxicity at inner hair cell-auditory nerve connections, loss of auditory nerve, and central auditory changes including overstimulation and deafness-induced plastic changes

Murugasu 1997). This negative feedback gain control can reduce the effects of traumatizing noise stimulation (Rajan 2001a, b). However, this medial efferent feedback system does not contribute to the development of acquired resistance (Canlon et al. 1988) to acoustic trauma (Yamasoba and Dolan 1998). Efferent feedback also slows the effects of aging (Liberman et al. 2014). The middle ear muscles do not contribute to the development of acquired resistance (Ryan et al. 1994).

7.1.2 Causes of TTS: Hair Cells

As work by Hudspeth, Flock, Corey, and others (e.g., Hudspeth 1989; Flock and Orman 1983, Pickles and Corey 1992 for reviews) progressed to show the role of hair cell stereocilia in transduction, this also pointed to a role in TTS, with TTS appearing from a noise-induced dysfunction in stereocilia-related mechanisms (e.g., Canlon et al. 1987; Saunders et al. 1986a, b; Saunders and Flock 1986; Thorne et al. 1986). This generally falls under the category of "mechanical" causes. Tip-links between stereocilia were first identified by Pickles (Pickles et al. 1984) and then associated with the transduction channels (see Hackney and Furness 2013 for review). These tip-links appear to be very sensitive to noise overstimulation and can be lost with excessive stimulation (e.g., Pickles et al. 1987, Sakaguchi et al. 2009). If the tip-links are lost, then the transduction channel can no longer be opened. The loss of tip-links in OHCs diminishes the "amplifier" effect of outer hair cell motility; with tip-link loss there can be a 20–60 dB threshold shift. The loss of tip-links in IHCs reduces or prevents events leading to transmitter release and auditory nerve excitation, yielding an even greater threshold shift. The lost tip-links appear to be capable of rapid regeneration if and when the stereocilia maintain their rigidity and orientation (e.g., Zhao et al. 1996; Indzhykulian et al. 2013). Stereocilia have a specialized actin organization that is responsible for maintaining rigidity, and this actin organization can become disrupted (depolymerized) by a noise-induced mechanical stress (Tilney et al. 1982, Tilney and Saunders 1983). When this happens, the stereocilia become more flaccid (e.g., Canlon et al. 1987; Saunders et al. 1986a, b, Saunders and Flock 1986; Thorne et al. 1986) disrupting the transduction process. The actin organization can be regained once the noise stops. Restoration of stereocilia rigidity and orientation then allows for tip-links to be regenerated and relinked to the transduction channels. This whole process takes longer to occur than when only regeneration of tip-links is necessary, and therefore the loss of stereocilia rigidity generates a longer threshold shift. There is a tipping point for the mechanical forces where actual damage to stereocilia occurs, and this can move the threshold shift from temporary to permanent.

Other chapters in this volume will discuss in detail how different stresses, including noise, can induce different forms of oxidative stress in different cell types in the cochlea (and elsewhere) and cause either a temporary disruption of function or induce cell death pathways and permanent threshold shift when cells are lost. This chapter will therefore not go into detail in this area and will only give the broad general picture while referring to the chapters with additional information. However, we do want to reemphasize that TTS can also result when metabolic/intracellular changes including energy depletion and oxidative stress are below the level to initiate apoptotic pathways but sufficient to interfere with the function of cells in the cochlea. For OHCs this could impair transduction, depolarization, and other processes that drive OHC motility and the amplifier function derived from this. For IHCs this impairs transduction and the processes leading to transmitter release. Hair cells can be directly affected or can be affected by free radical-induced changes in other cellular elements, such as supporting cells.

7.1.3 Causes of TTS: Supporting Cells

While supporting cells were once believed to be predominantly passive and structural in their mature cochlear function, they are now known to play important active roles in homeostasis and in generating and maintaining the environment necessary for proper and efficient hair cell function (e.g., Wan et al. 2013, Monzack and Cunningham 2013). These dynamic supporting cell functions include generating and maintaining the proper ionic composition of cochlear fluids, generating and maintaining composition of various matrices and the reticular lamina, clearance of excess synaptic glutamate, managing ion diffusion, as well as more general waste management and "cleansing" across the cochlear environment. Supporting cells also secrete maintenance factors, such as NTF3, that can influence the status of hair cells, auditory neurons, and their connections. Recent studies show that supporting cells can secrete heat shock proteins upon stress which could then have a protective influence on hair cells (May et al. 2013).

Both noise-related mechanical stress and the noise-induced buildup of free radicals can disrupt supporting cell functions. It is not yet completely understood how disruption of supporting cells causes TTS. There can be buckling of supporting cells (predominantly pillar cells) in animals under noise exposure conditions that produce a TTS, and this could have the effect of decoupling stereocilia, and that would change basilar membrane mechanics and other cochlear dynamics (Nordmann et al. 2000; Harding and Bohne 2004). Interestingly, a wide variety of stresses, including noise and ototoxic drugs, have been found to induce both an earlier appearance and a higher level of free radicals in supporting cells compared to IHCs and OHCs (e.g., Henderson et al. 2006; Jiang et al. 2005; Ohinata et al. 2000, 2003; Yamashita et al. 2004). TTS can appear under conditions in which the predominant increase in free radicals occurs in supporting cells rather than hair cells. Despite this greater induction of free radicals in supporting cells compared to hair cells, it is rare to find the loss of supporting cells prior to the loss of hair cells. This suggests that supporting cells have a large potential for recovery and that the loss of supporting cell functions may jeopardize hair cells and their function. That the effects of noise on supporting cells may jeopardize hair recovery, leading to PTS, is discussed in additional detail later in this chapter.

7.2 Causes of TTS: Inner Hair Cell-Auditory Nerve Connections as a Target

The current definition of TTS as having no lasting harmful effects has now been challenged by recent studies from Kujawa and Liberman (2006, 2009). These studies found that even when a noise does not produce any loss of hair cells or permanent change in the sound-evoked ABR threshold, there can still be permanent neuronal changes and functional consequences which specifically include reduced

suprathreshold ABR amplitude; i.e., the ABR is smaller at high sound levels, suggesting fewer neurons are responding to that sound. The overstimulation of the IHCs from noise or other stresses can cause excessive release of glutamate leading to excitotoxicity, with the swelling, bursting, and regression of some of the auditory nerve peripheral processes that had been connected to the IHC. Pujol and Puel (Puel et al. 1998; Pujol and Puel 1999) found that there is potential for lost processes to regenerate and reconnect to IHCs and suggested this might be a component of TTS. They also found recovery of suprathreshold response functions. The studies from Kujawa and Liberman (2006, 2009), however, show that such reconnection can be inefficient under their noise exposure conditions. Without reconnection there is eventual degradation and cell death of the auditory neuron. Moreover, the lost inner hair cell-auditory nerve (IHC-AN) connections tend to fall predominantly in one category of auditory nerve fibers, the low spontaneous rate group (Furman et al. 2013). Their loss then reduces the suprathreshold auditory brain stem responses. The apparent threshold may then recover, but there is a permanent loss of auditory neurons, and there might also be a reduced dynamic range. Regeneration and reconnection have been shown following noise overstimulation in guinea pig (Liu et al. 2012; Shi et al. 2013), and so auditory neuropathy following TTS noise may be variable depending on noise exposure conditions and species.

7.2.1 Strategies for Prevention and Repair

While many of the changes described above can occur independently, they can also occur simultaneously, with dominance depending on characteristics of the noise exposure. The timing for the different mechanisms and their effects can be different. One might expect that the most immediate effect would be from the mechanical influences of noise on the hair cell stereocilia and their tip-links which might in turn rapidly recover. Any effect through free radicals may depend on the cellular target (hair cell, supporting cells, auditory nerve, or other cochlear elements) and would be dependent on the rate of free radical formation and oxidative stress. It may be slower and longer lasting.

One immediate question is whether a true TTS should even be a target for prevention. Indeed, the two reflexes (middle ear and medial efferent) may have evolved for the purpose of providing a TTS for the purpose of protection from PTS. The loss of tip-links and/or loss of stereocilia stiffness could also provide valuable protection by stopping transduction and halting the progression from TTS to PTS. However, there are circumstances, such as for those serving in the military or in certain work situations, where even a brief temporary loss of hearing and communication can and often does put them at increased risk for survival (see reviews by McIlwaine 2009; Grantham 2011; Yankaskas 2013). Clearly, prevention of TTS could have value under the appropriate circumstance. In addition to the prevention of functional deficits, preventing the accumulation of free radicals in hair cells and/or supporting cells might also reduce free radical formation and TTS to a low level from which

recovery is possible (TTS) instead of allowing free radical formation and TTS to proceed to a higher level where cell death occurs. Preventing excessive accumulation of free radical could not only prevent TTS but also prevent the progression from TTS to PTS. The TTS which results from accumulation of free radicals in hair cells and/or supporting cells is currently a major target for testing therapeutics. This is discussed in Chap. 9 by Le Prell and Lobarinas. The noise-induced loss of IHC-auditory nerve connections of low spontaneous rate auditory nerve fibers also points to a need for therapeutic interventions designed to prevent this loss. Perhaps anti-excitotoxicity agents, or agents that induce a more efficient regeneration or reconnection, will be the most effective drugs for this purpose.

7.2.2 Noise-Induced Permanent Threshold Shift

Noise-induced permanent threshold shifts are generally associated with either cell death or with a permanent loss of cell function in remaining cells. The dysfunctional cells can be destined for cell death at a later time, even if present at the time of assessment, but can also remain in a dysfunctional state for long periods of time or even indefinitely. While a noise-induced cell loss causing permanent threshold shifts is most often "sensorineural" from the loss of hair cells and/or loss of auditory nerve, there can also be a loss of non-sensorineural elements. For example, noise can cause the loss of type IV fibroblasts that influence cochlear mechanical processes, and their loss can therefore result in less efficient transduction and threshold shifts (Adams 2009; Chap. 3 by Ohlemiller) without any loss of hair cells or auditory nerve. Sensorineural loss can be secondary to "intermediate steps" involving cell death in other cochlear elements including lateral wall and stria that can lead to changes (e.g., in endolymphatic potential or ionic transport) that can induce indirect loss of hair cells or auditory nerve (Chap. 3). However, the authors are not aware of any reports of finding a noise-induced loss of supporting cells in the organ of Corti without any adjacent loss of hair cells, despite supporting cells generally being the first site of free radical formation following stresses such as noise, ototoxins, and hypoxia.

7.3 Mechanisms Underlying Cell Death

Noise-induced cell death can be from apoptosis, a programmed cell death under the control of specific intracellular molecular pathways (see Chap. 19), or from necrosis, a more passive, less controlled mode (Yang et al. 2004). Necrosis is most often associated with mechanical damage to or within cells resulting in the cell or cellular elements swelling, breaking up, or rupturing. An inflammatory response is often associated with necrosis. There is often considered to be a "critical level" for noise exposures above which mechanical damage begins (Henderson et al. 1994; Henderson and Hamernik 2011, Wang et al. 2002). The critical level can be influenced by

multiple factors including the length of time of the noise exposure, the intensity of the noise exposure, and the type of noise (e.g., continuous versus impulse).

The same exposure intensity/duration can cause hearing loss in one subject and not in another similar subject, both in people and animal models, a topic explored in detail by Spankovich in Chap. 6. The amount of hearing loss can have large variability among subjects. There could be genetic or epigenetic effects through multiple genes that directly or indirectly influence sensitivity and susceptibility (see Chaps. 8, 14, and 17). These could influence the strength/efficiency of endogenous protective pathways such as the heat shock proteins or the levels of endogenous antioxidants. Variations, including heat shock protein-associated genes, have been found in genotyping studies comparing human populations with and without hearing loss (Konings et al. 2009; Sliwinska-Kowalska and Davis 2012, Sliwinska-Kowalska and Pawelczyk 2013).

Preconditioning or "toughening" can also come into play where previous low-level, non-damaging noise exposure(s) or other previous stresses to the cochlea can protect from a later potentially more damaging noise exposure (see Niu and Canlon 2002 for review). While a role for the middle ear or medial efferent reflexes has been ruled out (Ryan et al. 1994; Yamasoba and Dolan 1998), other endogenous protective mechanisms may be involved. Preconditioning may increase resistance to noise-induced oxidative stress by effects on endogenous levels of free radical scavengers/antioxidants (e.g., Harris et al. 2006, Yamasoba et al. 1998). It may also involve other stress pathways such as those involving heat shock proteins. Prior induction of heat shock proteins has been shown to provide protection from noise (Yoshida et al. 1999), and a low-level noise preconditioning will upregulate heat shock proteins and provide protection from an aminoglycoside ototoxicity (Roy et al. 2013). Conditioning may also be generated from stress pathways of the hypothalamic-pituitary-adrenal axis, involving glucocorticoid receptors and corticosteroids (Tahera et al. 2007). Other potential factors include changes in the presynaptic region of hair cells (Canlon et al. 1993), alterations in F-actin distribution in supporting cells and hair cells (Hu and Henderson 1997), and a decrease in calbindin D-28 immunoreactivity in OHCs (Canlon 1996).

Mechanical forces from the noise exposure can act at an individual cell level but can also act on the general structure of the cochlea. Mechanical stress can result in the breaking of junctions separating cochlear fluids and induce cell death in the region of leakage by ionic poisoning of cells (e.g., Ahmad et al. 2003). Mechanical disruption of individual cells can range from bursting and rupturing of entire cells to effects that are limited to specific cellular or intracellular components, such as F-actin in the cuticular plate (Hu and Henderson 1997). For hair cells, the stereocilia are particularly sensitive to noise, and there can be a gradient in the effect and damage to the stereocilia depending on the force and duration of the noise. At the low end of the gradient is the loss of tip-links and actin structure that can recover, giving TTS, next would be a permanent loss of microstructure organization leading to dysfunction and cell death by apoptosis or active removal by scar formation, greatest would be a physical loss and rupture leading to hair cell death necrosis.

There appears to be a more limited ability for cochlear hair cells to repair themselves compared to hair cells in vestibular end-organs. One potential explanation is "trigger-happy supporting cells" eager to begin the process of scar formation as a protective mechanism. Raphael et al. (1996) suggested that supporting cells are programmed to maintain the barrier between endolymph and perilymph and can respond to cues (or lack of cues) from hair cells that have some level of damage or dysfunction. The surrounding supporting cells respond by forming a new tight junction, the scar, in the process choking and killing the noise-damaged hair cell before it has a chance to repair and recover. More often, scar formation is part of an orderly program as a hair cell goes through the controlled progression of cell death following one of several intracellular molecular pathways during apoptosis.

Noise-induced cell death by apoptosis is most often the consequence of stress-induced processes that can be induced by free radical formation/oxidative stress and/or energy depletion/mitochondrial changes. There is a gradation/continuum in effect depending on the amount, type, and duration of noise stress and resulting amount of free radical formation and energy depletion. There can be immediate and delayed effects. Studies have shown free radical formation beginning during noise exposure or several days after the noise exposure (Yamashita et al. 2004). Histopathology studies show that the margins of the region of cell death continue to expand well after the noise exposure (Hu 2012 for review). This suggests different mechanisms and interactions among cellular elements (see Sect. 7.3.1) and also influences approaches for therapeutics for prevention and repair.

7.3.1 Role of Supporting Cells in PTS

Studies examining free radical formation following noise in the organ of Corti show that cells of the lateral wall and then supporting cells are among the first cochlear elements to demonstrate increases in free radicals following the noise overstimulation and these non-sensory cells also often show the highest levels of free radical increases (Chap. 3; see also Yamashita et al. 2004). Nonetheless, hair cells can move to noise-induced apoptosis even when supporting cells do not, suggesting that the threshold to induce apoptotic pathways is lower in hair cells. Supporting cells may also be more capable of repair or using endogenous protective pathways to block apoptosis. Even if apoptosis does not manifest in the supporting cells, free radical formation and energy depletion can decrease their function. Supporting cells have an active role in generating and maintaining the environment for efficient transduction as well to support the health of other cochlear elements (Wan et al. 2013, Monzack and Cunningham 2013), with their junctional complexes essential for K+ transport and homeostasis. Less efficient supporting cell function following noise could compromise the ability of hair cells to withstand stress and compound the direct effect of noise of hair cells. It could also explain the later occurring effects on hair cells, if supporting cells go through a period where they are less efficient at maintaining the cochlear environment.

7.3.2 Approaches for Protection: Therapies

Mechanical changes offer less opportunity for pharmacological therapeutic interventions for protection or repair, although the heat shock protein pathway can act against protein folding and enhancing this pathway could protect or repair such changes. When mechanical stress induces apoptosis, the pathway can be blocked (e.g., Bielefeld et al. 2011; Fetoni et al. 2014). Indeed, apoptosis offers the greatest opportunity for pharmacological interventions, and this has been the topic of multiple recent reviews, i.e., Abi-Hachem et al. (2010) and Poirrer et al. (2010). Protection could be achieved by reducing the initiating (upstream) factors, directly neutralizing free radicals through the use of free radical scavengers, or by reducing oxidative stress and/or energy depletion by enhancing intracellular protective pathways. Another approach is by negating or interrupting the cell death pathways. These are discussed in depth in Chap. 20 by Van de Water et al. Because there can be delays in free radical formation and apoptosis following noise (Yamashita et al. 2004), there is a window for protection from these processes during a period of days following noise, as well as opportunity to enhance repair (e.g., Yamashita et al. 2005). These approaches are relevant to drug-induced hearing loss (Chaps. 10 and 11), age-related hearing loss (Chap. 16), and hearing loss due to surgical trauma (Chap. 19).

7.4 Noise-Induced Effects on Central Auditory Pathways

Noise overstimulation will influence the mature central auditory pathways in several ways. There can be an immediate synaptic overstimulation from the noise. With glutamate as the excitatory transmitter in many central auditory synapses, there is a potential for excitotoxicity from this initial overstimulation. Evidence of excitotoxicity, however, is rarely found except in animal models such as gerbil where the formation of microcysts in the cochlear nucleus is influenced by ambient levels of noise (Statler et al. 1990). There are also central auditory changes that are part of the "normal" response to noise-induced increases in the level of auditory activity that are part of "active listening" (Fritz et al. 2005; Zion Golumbic et al. 2012 for reviews) and experience-associated auditory plasticity (Schreiner and Polley 2014 for review). Even as the noise overstimulation continues, the increased activity in the central pathways may be of short duration because of TTS- or PTS-related changes in the cochlea that will diminish the activity in the auditory nerve and reduce the overstimulation, often to under-stimulation. The duration of the understimulation or "deafness" would be related to the duration of the TTS or appearance of PTS. The under-simulation or deafness may only be across a restricted frequency range, with tonotopic regions and divisions influenced in different ways. Deafness-associated changes might then be expected to occur.

There is a large literature on deafness-associated changes to the central auditory pathways. These can be brief and reversible or permanent, depending on the

characteristics of the noise overstimulation and its effects in the cochlea. As with overstimulation, deafness can induce central auditory changes that are part of the normal sound processing in active listening and experience-dependent plasticity. Depending on the type and duration of the noise-induced deafness, these central auditory changes may not reverse in the "normal" fashion and may persist and induce other changes that are not normally occurring. Such "maladaptive changes" may contribute to the generation of tinnitus (Wang et al. 2011a, b for review) as discussed later in this section. Deafness-associated central auditory changes can involve up- and downregulation of neurotransmitters, receptor subunits, ion channels, and other synaptic elements that change the balance between excitation and inhibition as well as neuronal excitability and intrinsic properties (see Altschuler et al. 2004; Caspary et al. 2008; Dong et al. 2009 for reviews). Changes in the inhibitory amino acids GABA and glycine and in GABA receptor subunits appear to be common deafness-induced changes (Caspary et al. 2008; Dong et al. 2009, Asako et al. 2005; Buras et al. 2006). There can also be deafness-associated changes in glutamate receptors (Sato et al. 2000 for review). Ion channels can change phosphorylation state as a consequence of changes in auditory activity (Brown and Kaczmarek 2011). Many ion channels have deafness-associated changes in expression including the two-pore domain potassium channels (Holt et al. 2006; Cui et al. 2007). The degree of deafness required to produce central auditory changes may vary for each different type of change.

There can also be a loss of auditory nerve connections in the cochlear nucleus secondary to loss of IHC-auditory nerve connections and the subsequent loss of auditory neurons (e.g., Kim et al. 2004a, b). The loss of auditory nerve terminals to cochlear nucleus neurons can then induce reactive changes. Growth cone markers (Gap-43) can be found in the cochlear nucleus following acoustic trauma (Michler and Illing 2002) as well as ultrastructural evidence of new synapses (Kim et al. 2004a). The loss of auditory nerve terminals can lead to increases in somatosensory (Shore 2011 for review), glycinergic (Asako et al. 2005), and/or cholinergic (Michler and Illing 2002) terminals in the CN. In addition there can be changes in glial elements and neurotrophic factors (e.g., Feng et al. 2012; Lurie and Rubel 1994). There are also changes in surviving auditory nerve synapses, including the evoked release of transmitter (Wang et al. 2011a, b).

Deafness-associated changes are seen all along the ascending and descending central auditory pathways from the cochlear nucleus to the auditory cortex and both noise. Deafness can also influence associated nonauditory pathways such as the limbic system (Krause and Canlon). Because effects can be limited to specific tonotopic divisions within the auditory pathways, auditory regions with underactivity can lose space to regions with normal activity (e.g., King and Moore 1991).

There is currently increased interest in the influence of noise on the central auditory system because noise overstimulation is being used in many animal models of tinnitus. It is believed that many of the central nervous system changes listed above can contribute to the generation of tinnitus. Noise-induced tinnitus is complex and may occur in several phases. An initial phase may be the effects of noise in the cochlea and either a temporary or permanent loss of IHC-auditory nerve

connections (Hickox and Liberman 2014; Singer et al. 2013). Changes in the cochlear nucleus and auditory brain stem, including those listed above (Baizer et al. 2012; Richardson et al. 2012; Shore 2011; Wang et al. 2011a, b, for reviews), may be the next phase causing increased activity in the auditory brain stem (Kaltenbach 2011 for review). This can then lead to adaptive plasticity and further changes in higher centers (Knipper et al. 2013, Eggermont 2008; Roberts et al. 2010 for reviews) and associated pathways (e.g., Engineer et al. 2013; Kraus and Canlon 2012). Noise-induced changes in the cochlea and the central nervous system may then be a target and strategy for prevention or treatment of tinnitus.

7.5 Conclusions

Noise can impact the auditory system along a continuum ranging from mild disruption of hair cell stereocilia resulting in a brief TTS to a large mechanical damage of cochlear elements resulting in a PTS and profound deafness. Noise can also induce tinnitus, with or without accompanying PTS. Different underlying mechanisms can come into play along this continuum, both mechanical and metabolic and via different intracellular pathways. As mechanisms have been uncovered, this has led to the testing of mechanism-based therapeutic interventions for protection, recovery, and/or repair. The metabolic mechanisms that have been uncovered currently offer the best targets for therapeutic interventions. Free radical-induced damage and apoptotic pathways have been the most targeted mechanisms, since pharmacological interventions have been developed that can reduce free radical accumulation or interrupt apoptosis. Both animal studies and clinical trials directed toward these mechanisms are now under way that could provide effective therapeutics in the near future. Natural protective mechanisms, such as stress pathways or neurotrophic factors, could also provide targets for interventions, but these have not yet moved beyond animal studies and would have a longer time line before they could be applied clinically.

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Chapter 8
Oxidative Stress in Noise-Induced Hearing Loss

Daisuke Yamashita

8.1 Introduction

8.1.1 Background

Hearing loss is the greatest cause of sensory disability. Global Burden of Disease 2010 estimated that 1.3 billion people are affected by hearing loss, and investigators rated hearing loss as the thirteenth most important contributor (19.9 million years, 2.6 % of total number) to the global years lived with disability (YLD). The World Health Organization (WHO) estimates that 10 % of the world population is exposed to sound pressure levels that may cause noise-induced hearing loss (NIHL) with continued exposure (Basner et al. 2013).

NIHL is a leading occupational health risk in industrial societies and military settings and is one of the most common forms of sensorineural hearing impairment, second only to presbycusis (age-related hearing loss: ARHL). About 22 million US workers are exposed to potentially hazardous noise levels at their work site and, annually, an estimated US$242 million is spent on compensation for hearing loss disability (NIOSH 2013). It is estimated that worldwide 16 % of all hearing impairments are due to exposure to loud noises (Nelson et al. 2005). However, it is very difficult to know the exact number of people at risk, because, in addition to the traditional risks of industrial work and the military, the recent increasing use of iPods and other MP3 players and attending discos and rock concerts have added to this risk.

Recognition that hearing loss results from noise exposure dates back at least as far as Ramazzini's (1713) classic occupational medicine treatise "*De Morbis Artificum* (Disease of Workers)" (Am J Public Health. 2001). Ramazzini recognized

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that exposure to both occupational and environmental noise can lead to hearing loss in individuals and entire populations. However, susceptibility to the damaging effects of noise differs remarkably among individuals. NIHL has been therefore classified as a complex disease, reflecting the interaction of genetic and environmental factors. Understanding the molecular pathways and genes that underlie this hearing loss is critical to design rational preventive and possible treatment strategies.

8.1.2 Mechanisms of Noise-Induced Hearing Loss

The mechanisms of NIHL can be classified into two main categories: (1) direct mechanical damage and (2) intense metabolic damage (Schuknecht 1993; Borg et al. 1995; Duan et al. 2002; Wang et al. 2002). First, the direct mechanical damage of noise itself leads to both mechanical disruption of the stereocilia and direct damage to sensory and supporting cells. Mechanical destruction of the hair cells and supporting structures has been long known to result in NIHL (Spoendlin 1971; Hunter-Duvar and Elliott 1972, 1973; Hamernik and Henderson 1974; Hamernik et al. 1974; Hunter-Duvar and Bredberg 1974; Hawkins et al. 1976; Hamerick et al. 1984). From statistical (epidemiological) studies, it has been possible to define the level at which a noise level and exposure time is likely to yield NIHL with some probability, although there remains considerable controversy about the precise boundary above which noise is considered to be potentially hazardous. Given this, hearing conservation programs vary from country to country. Despite the introduction of standards requiring hearing protection, reduction in occupational noise exposure in developed countries (i.e., "Buy Quiet" programs), and extensive public health efforts, there are still a number of individuals working in construction, the military, agriculture, and other occupations where conventional programs are difficult to operate or ineffective because of the very large variability in sensitivity and challenges with compliance.

The second major contributing mechanism of NIHL is metabolic damage via oxidative stress, with associated excitotoxicity, compromised blood flow, and alteration of ion homeostasis. Cochlear mechanosensory hair cells respond to noise stress by generating reactive oxygen species (ROS) and reactive nitrogen species (RNS). High ROS and RNS levels can overwhelm cellular antioxidant defenses, causing oxidative damage to DNA, lipids, and proteins, as well as subsequent hair cell death and hearing loss (Ohlemiller et al. 1999a; Simon et al. 2000; Poirrier et al. 2010). These are reviewed in detail in Chaps. 2–4.

8.2 Oxidative Stress and Free Radicals

Most living organisms need oxygen for their survival. Oxygen creates ROS such as the hydroxyl radical ($^{\bullet}OH$), superoxide radical ($O_2^{\bullet-}$), and hydrogen peroxide (H_2O_2) (Halliwell and Cross 1994; Mates et al. 2012; Farrugia and Balzan 2012; Sinha et al. 2013). Leakage of electrons during the electron transport to the ultimate electron

acceptor leads to binding to oxygen (O_2) and is the main source of ROS. There are in addition a number of RNS derived from nitric oxide (NO). Of these, peroxynitrite ($ONOO^{\bullet-}$), a highly reactive species derived from NO and $O_2^{\bullet-}$ (Arteel et al. 1999), is extraordinarily reactive and potentially the most harmful radical (Crow and Beckman 1996). While free radicals play an essential role in maintaining the physiological condition of the body, excess accumulation of ROS and RNS can damage basic components required for cell function and survival (Nanetti et al. 2011).

Oxygen itself in the molecular form (O_2) possesses a unique electronic configuration and can act as a radical. By accepting one electron, the molecular oxygen forms the superoxide anion radical ($O_2^{\bullet-}$), a relatively stable intermediate (Orrenius et al. 2007; Miller et al. 1990). Superoxide anion radical in the body can arise either from metabolic processes or following activation of oxygen by irradiation. This superoxide anion is considered as the primary ROS (Valko et al. 2007) and interacts with other molecules through enzyme- or metal-catalyzed processes to produce secondary ROS (Valko et al. 2005).

Redox homeostasis is maintained in an organism as equilibrium between antioxidant and oxidant levels (Dasuri et al. 2013). Damaging ROS and RNS are formed as unavoidable by-products of metabolism but their damaging effects are normally counteracted by endogenous antioxidant enzymes (e.g., catalase, superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione reductase) and other antioxidant substances (e.g., glutathione (GSH), metallothionein, vitamin A, vitamin C, and vitamin E) (Duffy et al. 1998; Suemori et al. 2006; Vatassery 1998). However, oxidative stress represents a state in which these antioxidant defenses are overwhelmed and no longer capable of protecting the cell from oxidative damage. Accumulation of ROS/RNS can result in a number of detrimental effects such as lipid peroxidation, protein oxidation, and DNA damage (Smith et al. 2013). Substantial evidence indicates that oxidative stress is a major contributor to the pathophysiology of a variety of neurodegenerative disorders including not only sensorineural hearing loss but also a number of other acute central nervous system (CNS) injuries and progressive degenerative disorders.

8.3 Oxidative Stress and Noise-Induced Hearing Loss

A number of studies have shown increased ROS and RNS and toxic free radical formation during and after intense noise (for detailed review see Le Prell et al. 2007). Lim and Melnick (1971) first suggested a relationship between noise-induced inner ear pathology and metabolic activity. Thalmann et al. (1975) subsequently reported that the cochlea operated at a high level of metabolism. The mitochondrial electron transport chain has been recognized as a major source of ROS, and the high energy demand are supported by the stria vascularis (Wangemann 2002). Yamane et al. (1995) observed increases in superoxide ($O_2^{\bullet-}$) along marginal cells of stria vascularis, along with empty strial capillaries, after an exposure to intense noise (120–125 dB SPL). Ohlemiller et al. found increases in the hydroxyl radical ($^{\bullet}OH$) after intense noise (110 dB SPL). Also, they described in mice the influence of SOD

and GPx, which are two important enzymes of the cochlear antioxidant defense system (Ohlemiller et al. 1999a, b, 2000; McFadden et al. 2001). Nicotera et al. (1999) and Henderson et al. (2006) demonstrated that ROS formation also occurs in outer hair cells (OHCs).

The cochlear vascular system plays a significant role in NIHL. Cochlear blood flow (CBF) is controlled by several factors under normal condition, including systemic changes (Miller and Dengerink 1988), sympathetic influence (Laurikainen et al. 1994), and local autoregulation (Miller et al. 1995; Konishi et al. 1998). While many factors influence CBF, Thorne and Nuttall (1987) reported that intense noise exposure reduced CBF by using laser-Doppler measurement (Thorne and Nuttall 1987), and Quirk et al. (1992) demonstrated the significant reduction of both red blood cell velocity and diameter of vessels at the stria vascularis using intravital microscopic measurement. Ohinata et al. (2000) demonstrated that intense noise induced formation of vasoactive lipid peroxidation products in the organ of Corti and lateral wall. The decrease in CBF leads to ischemia/reperfusion injury in the inner ear. First, ischemia in the cochlea results in deficiency of oxygen with the mitochondria, leading to generation of free radicals. Second, the increase of CBF with reperfusion conversely results in another burst of free radical generation (Lamm and Arnold 1996; Latoni et al. 1996). Miller et al. (2003) reported that a by-product of free radical-induced lipid peroxidation (8-isoprostaglandin F2a) can lower CBF and blocking the 8-isoprostaglandin F2a receptor can inhibit the noise-induced reduction of CBF. These results demonstrate a mechanism by which ROS reduces CBF during intense noise (for additional discussion, see Le Prell et al. 2007).

Glutamate is the excitatory neurotransmitter that acts at the synapses of the inner hair cells (IHC) with VIIIth nerve afferent fibers. During intense noise, the IHC are highly active, leading to the release of large amounts of glutamate into the synaptic cleft (Henderson et al. 2006). The levels of glutamate in the cleft can overstimulate the glutamate receptors on the postsynaptic cells. The result is the condition of excitotoxicity, characterized by swelling and sometimes bursting of the postsynaptic cell dendrites (Kandel et al. 2000). The swelling is a result of postsynaptic ion and water influx into the VIIIth nerve terminals that occurs due to excessive excitation (Pujol et al. 1990). Massive glutamate efflux in the perilymph of the cochlea is observed after intense noise (Hakuba et al. 1997, 2000). Besides this excessive release, the failed uptake of glutamate from the synaptic clefts into surrounding cells also contributes to excitotoxicity. Furness et al. (2009) demonstrated that glutamate aspartate transporter (GLAST), which is one of Na^+-dependent glutamate transporters, is present in the supporting cells surrounding IHCs. Glutamate–cysteine ligase (GCL) is the rate-limiting enzyme for GSH synthesis. The expression of GCL is mediated by activator protein-1 (AP-1) and nuclear factor-kappa B (NF-kappaB), which are known to participate in stress-induced apoptotic pathways in neuronal cells. Nagashima et al. (2007) demonstrated that intense noise exposure (4 kHz OBN, 125 dB SPL, 5 h) facilitates the expression of GCL catalytic subunit in the cochlea possibly through the activation of transcription factors including AP-1 and NF-kappaB. Glutamate excitotoxicity, one of the contributing factors in noise-

induced oxidative stress, can be reduced by the action of cochlear *N*-methyl-D-aspartate (NMDA) receptors using carbamathione, which act as glutamate antagonists (Kopke et al. 2000). They demonstrated that noise-induced threshold shifts and sensory hair cell losses were reduced in carbamathione-treated adult chinchilla, compared to control subjects.

Several recent investigations further elucidate the mechanism between NIHL and oxidative stress. Peroxiredoxins are components of a ubiquitous thioredoxin-dependent antioxidant defense system that catalyzes the inactivation of ROS, including hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^{\bullet-}$), as well as the reduction of protein sulfhydryl groups (Chae et al. 1999, 2012). Prx3, one of six isoforms of the family of mammalian peroxiredoxins, is a mitochondrion-specific enzyme required to maintain normal mitochondrial metabolism and integrity (Wonsey et al. 2002). Chen et al. (2013) investigated the role of Prx3 in hair cell death induced by intense noise. In vivo, Prx3 transiently increased in mouse cochlear hair cells after traumatic noise exposure; when Prx3 declined, hair cell loss began. Conversely, reducing Prx3 levels with Prx3 siRNA increased the severity of noise-induced trauma. They suggest that Prx3 is upregulated in response to oxidative stress and that maintenance of Prx3 levels in hair cells is a critical factor in their susceptibility to NIHL. In addition, nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NADPH oxidase: NOX) are enzymes that transport electrons across the plasma membrane and generate superoxide radical from molecular oxygen. The NOX family often plays a role in host defense through ROS dependent bacterial killing (Krause 2004; Bedard and Krause 2007). In the central nervous system, NOX activity is required for neuronal signaling, memory, and central cardiovascular regulation, while overproduction of ROS by NOX contributes to neurotoxicity, neurodegeneration, and blood–brain barrier damage in experimental stroke (Infanger et al. 2006; Kahles et al. 2007). Vlajkovic et al. (2013) have investigated the expression and distribution of NOX/DUOX (DUOX: dual oxidase) members of the NADPH oxidase family (NOX1-5 and DUOX1-2) in the rat cochlea and their regulation in response to noise. They demonstrated that noise-induced upregulation of NOX1 and DUOX2 could be linked to cochlear injury. In contrast, downregulation of NOX3 may represent an endogenous protective mechanism to reduce oxidative stress in the noise-exposed cochlea.

8.4 Continuing Oxidative Stress After Intense Noise and Clinical Application of Post-exposure Treatment

Fredelius et al. (1988) initially reported that OHC damage develops continuously for several days after acoustic overstimulation. Though the damage in the OHCs was restricted in guinea pig cochleae immediately after noise exposure, the number of missing OHCs increased at 24 h after intense noise. The damage clearly continues to increase out to at least 14 days post-noise (Yamashita et al. 2004a), and may continue for longer periods. Henderson et al. (1994) observed delayed degeneration

of OHCs continuing up to 30 days following impact noise in chinchillas. Depending on species, noise condition (intensity, frequency, and exposure time), and individual sensitivity to noise, the extent of damage and time varies. Because direct mechanical damage presumably occurs only during the sound exposure, it is possible that the metabolic damage is associated with the gradually progressing cell death following noise exposure. Consistent with ongoing metabolic stress driving delayed cell death, Yamashita et al. (2004a) demonstrated continued free radical formation for 10–14 days after intense noise, including both immunoreactivity to 4-hydroxy-2-nonenal (4-HNE) and nitrotyrosine (NT) as histochemical makers of ROS and RNS formation, respectively. In this study, free radical formation began laterally in the supporting cells of the organ of Corti and gradually increased in the hair cell, reaching a maximum at 7–14 days following the exposure. These results accord with gradual spread of OHC damage morphologically.

Subsequently, Yamashita et al. (2005) reported that treatment with salicylate and vitamin E initiated up to 3 days after noise exposure significantly attenuated the functional and morphological consequences of noise trauma, although pretreatment or treatment within 24 h of the exposure was most effective. Consistent with the involvement of ROS and RNS in NIHL, the expression of 4-HNE and NT, was reduced in cochlear cells using this antioxidant combination therapy. Campbell et al. (2011) report post-noise protection using the antioxidant D-methionine, although protection decreased across the first 7-h post-noise. Kopke et al. (2000) have also reported that treatment with the antioxidant N-acetylcysteine (NAC) delivered immediately after intense noise reduced hearing impairment, although less effectively than pretreatment. Finally, Choi et al. (2011) showed that administration of antioxidant drugs (4-OHPBN plus NAC plus ALCAR) extended up to 10 days after noise exposure can effectively treat acute acoustic trauma in chinchilla model, even when treatment is delayed 24–48-h post-noise.

Though variation of drugs, dose, timing, duration of treatment, species of subjects, and duration and intensity of noise exposure are clearly important factors influencing effectiveness of treatment, these results are clinically important in helping to define the "window of opportunity" for the prevention of NIHL. These findings and their implications for treatment are in good agreement with the observations that antioxidants reduce tissue injury and cell death in animal models of focal cerebral ischemia and traumatic brain injuries.

8.5 Genetic Factors Influencing Oxidative Stress and NIHL Susceptibility

Many researchers have investigated genes that may influence NIHL. Yamashita et al. (2008) reported that Bcl-2 genes regulate NIHL. They showed that Bcl-xL, the anti-apoptotic Bcl-2 gene, was robustly expressed in OHCs following TTS exposure, whereas Bak, the pro-apoptotic gene, was expressed following PTS exposure. These results indicate an important role of Bcl-2 family proteins in regulating

sensory cell survival or death following intense noise. Niu et al. (2003) also showed that Bcl-2 acts as an inducible neuroprotective gene that is upregulated by sound conditioning. Vicente-Torres and Schacht (2006) have reported that excess calcium in CBA/J mice OHCs following noise exposure (broadband noise: 2–20 kHz) triggers mitochondria-mediated death pathways through calcineurin-mediated activation of Bcl-2-associated death promoter (BAD). Yamashita et al. (2004b) demonstrated that intense noise (PTS exposure) induces an increase in cytosolic AIF (apoptosis-inducing factor), where it can function as a free radical scavenger, while endonuclease G (EndoG) translocates to the nucleus, where it can induce caspase-independent cell death. These results reflect the critical balance of pro- and anti-apoptotic factors ongoing simultaneously that define survival or death in the cell following oxidative stress.

Genetic factors add to the complexity of these cell death mechanisms. Carlsson et al. (2005) reported that some individuals are more susceptible to NIHL than others. Heinonen-Guzejev et al. (2005) demonstrated in a twin study that noise sensitivity does aggregate in families. However, there is still limited information about genetic polymorphisms that may be involved in susceptibility to NIHL. Gong and Lomax (2012) provided a detailed review of multiple genes implicated in NIHL, some of which are associated with oxidative stress pathway, including catalase (CAT), Cu/Zn-superoxide dismutase (SOD1), Mn-superoxide dismutase (SOD2), paraoxonase2 (PON2), glutathione peroxidase 1 (GPX1), and glutathione S-transferase Mu (GSTM1). Additional discussion is provided in Chap. 17.

Catalase is a tetramer of four polypeptide chains and functions to catalyze the decomposition of hydrogen peroxide to water and oxygen. Jacono et al. (1998) showed that localization of catalase is much more in the organ of Corti than in the stria vascularis. Konings et al. (2007) investigated whether variations (single-nucleotide polymorphisms: SNPs) in CAT influence noise susceptibility in two independent noise-exposed populations (1,261 Swedish and 4,500 Polish). Their results indicated that significant interactions were observed between noise exposure levels and genotypes of five SNPs in the Polish population; two of these SNPs were also observed in the Swedish population.

Superoxide dismutase (SOD) is an enzyme that converts superoxide radicals to oxygen and hydrogen peroxide, which in turn is then metabolized to water by glutathione peroxidase or catalase. SOD, in mammals, constitutes three groups of distinct enzymes genetically and geographically. SOD1 is abundant and widely localized in the cochlea, including the organ of Corti, spiral ligament, stria vascularis, and spiral ganglion (Rarey and Yao 1996; Staecker et al. 2001). In mice lacking SOD1, there is enhanced susceptibility to NIHL compared to wild type as demonstrated by Ohlemiller et al. (1999a). Furthermore, the application of SOD1 attenuated NIHL (Seidman et al. 1993). However, the overexpression of SOD1 did not protect against NIHL (Coling et al. 2003). For detailed discussion, see Chap. 3.

Paraoxonases (PONs) are glycosylated proteins, which are a group of enzymes involved in the metabolism of organophosphates and lipid derivatives. The PON gene family consists of PON1, PON2, and PON3 on chromosome 7q21–q22 (Primo-Parmo et al. 1996). PON2 is a ubiquitously expressed intracellular protein

throughout the body that can protect cells against oxidative stress. Fortunato et al. (2004) have investigated the association between the susceptibility to NIHL and SOD2 and PON2 polymorphisms in workers from an aircraft factory, where they are exposed to prolonged loud noise (average 94 dB for 20 years). They demonstrated that the IVS3-23 T/G and IVS3-60 T/G SOD2 polymorphisms and PON2 (SC + CC) genotype are related to NIHL irrespective of age and smoking habits.

Glutathione peroxidase (GPx) functions biochemically to reduce free hydrogen peroxide to water and to reduce lipid hydroperoxides to their corresponding alcohols, which mainly protect the organism from oxidative stress. Glutathione *S*-transferase (GST) enzymes have been known to catalyze the conjugation of the reduced form of glutathione (GSH) to xenobiotic substrates for the aim of detoxification, which can constitute up to 10 % of cytosolic protein in human organs. GSTM1, which is one of 13 GST classes based on structure, is involved in regulating apoptosis pathways through protein–protein interactions. In Gpx1 knockout mice, ABR threshold was increased after intense noise and cell death of sensory hair cells and nerve fibers was also increased after intense noise relative to wild-type controls (Ohlemiller et al. 2000; McFadden et al. 2001). Data from human subjects suggest a similar phenomenon in humans. Rabinowitz et al. (2002) demonstrated that individuals possessing the GSTM1 gene had significantly better hearing at high frequencies compared to GSTM1-null individuals, in an epidemiological human study.

8.6 New Concept of "Oxidative Stress" Research

8.6.1 ROS Signaling

Free radicals, including ROS and RNS, in abundance clearly are toxic substances that induce nonspecific damage in various biological molecules. However, ROS biochemistry is now developing an emerging concept for physiological functions of ROS in the regulation of cell signal transductions. ROS signaling functions and their mechanisms are precisely regulated by several endogenous moderate electrophiles that are themselves generated from ROS during diverse physiological and pathophysiological cellular responses (for detailed discussion, see Chap. 2).

As an example of the contributions of ROS to normal cell physiology, H_2O_2 has been said to act as a potential signaling molecule that mediates vascular tone regulation (Matoba et al. 2000; Burgoyne et al. 2007). Furthermore, although the toxicity of nitric oxide (NO) is augmented by reactions with ROS, NO serves as a master cell signaling molecule involved in diverse biological phenomena (Schopfer et al. 2003). Akaike et al. (2013) demonstrated that protein S-guanylation induced by 8-nitro-cGMP may reveal new aspects of ROS- and NO-related redox chemical biology, physiology and pathophysiology, and pharmaceutical chemistry and may lead to development of therapeutics for oxidative stress-related diseases. Finally, Numajiri et al. (2011) reported that, in ischemic mouse brain, SNO-PTEN was found in the core and penumbra regions, but SNO-Akt, which is known to inhibit Akt activity, was found only in the ischemic core. These results indicate that inhibition of PTEN

activity in the penumbra would be expected to promote the cell survival through enhanced Akt signaling. On the other hand, SNO-Akt formation in the ischemic core would inhibit the neuroprotective pathway. The mechanism of ischemic insult leading to NIHL in the inner ear is similar to ischemic injury to the brain. Further investigation of "ROS signaling" at physiological levels may lead to better understanding of the role of ROS in normal function and contribute to the development of new strategies for more complete prevention in NIHL.

8.6.2 Programmed Necrosis "Necroptosis"

It has often been assumed that necrosis is distinct from apoptosis, based on the understanding that necrosis is not programmed by molecular events. It is now clear, however, that necrotic cell death can be driven by defined molecular pathways, that is, a type of programmed necrotic cell death called "necroptosis" (Cho et al. 2009; He et al. 2009; Vandenabeele et al. 2010; Zhang et al. 2009). Necroptosis is characterized by cell swelling, mitochondria dysfunction, plasma membrane permeabilization, and release of cytoplasmic content to the extracellular space. This form of cell death is also associated with high mitochondrial ROS production, and unlike apoptosis, it does not involve DNA fragmentation (Wu et al. 2012).

Although necroptosis may have evolved as a line of defense against intracellular infection (Cho et al. 2011; Kaiser et al. 2013), recent studies implicate it in a variety of disease states. Necroptosis is of central pathophysiological relevance in myocardial infarction and stroke (Degterev et al. 2008; Smith et al. 2007), ischemia–reperfusion injury (Linkermann et al. 2012; Oerlemans et al. 2012), inflammatory bowel diseases (Welz et al. 2011; Günther et al. 2011), and a number of other common clinical disorders. At the molecular level, intracellular assembly of a highly regulated complex, the necrosome, can be triggered by death receptors, including TNF receptor 1 (Oberst and Green 2011; Weinlich et al. 2011), cell-surface toll-like receptors (Kim and Li 2013; Seya et al. 2012), and DAI (which may act as a cytoplasmic viral RNA sensor) Welz and Pasparakis 2012; Upton et al. 2012). The continuing elucidation of the molecular subroutines of various forms of regulated necrosis, including necroptosis, and the efficient design of combination therapies hold promise for our ability to control regulated necrosis in clinical settings (Linkermann and Green 2014).

8.7 Conclusion

Many researchers have investigated the relationship between free radicals and various diseases, including cancer and autoimmune and neurodegenerative disorders. There is also clear support for the role of free radicals in NIHL as described in this chapter. And based upon these results, many strategies have been proposed to prevent NIHL, and promising results have been reported. However, it is still impossible

to completely prevent NIHL. One of the reasons why treatment is intractable is thought to be due to the delayed formation of free radicals which indicate that it is clinically important to continue therapy for some multi-day, or perhaps even multi-week, period following noise exposure. Clearly with pro- and anti-apoptotic factors simultaneously determining survival or death in the cell following oxidative stress, the mechanism of NIHL is certainly more complicated than we have in the past thought. Furthermore, individual genetic and epigenetic differences have strong yet undefined influences in both sensitivity to NIHL and the efficacy of proposed interventions to prevent NIHL. To effectively reduce NIHL across society, an increase in education on the use of hearing protective equipment in noisy situations continues to be very important, and the importance of education of adolescents and young adults to understand and choose safe listening levels on audio devices is receiving increasing attention. As described last in this chapter, the new concepts of "the physiological cell signaling function of ROS" and "programmed necrosis, necroptosis," may dictate new strategies of translational research and provide promise for more complete therapeutic treatment of NIHL.

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Chapter 9
Strategies for Evaluating Antioxidant Efficacy in Clinical Trials Assessing Prevention of Noise-Induced Hearing Loss

Colleen G. Le Prell and Edward Lobarinas

Abbreviations

ABR	Auditory brainstem response
ASHA	American Speech–Language–Hearing Association
BKB	Bamford-Kowal-Bench sentences
CST	Connected Speech Test
CHABA	Committee on Hearing, Bioacoustics, and Biomechanics
dB	Decibel
DoD	Department of Defense
DPOAE	Distortion product otoacoustic emission
EHF	Extended high frequency
FDA	Food and Drug Administration
HCoE	Department of Defense Hearing Center of Excellence
HINT	Hearing in noise test
HPD	Hearing Protection Device
Hz	Hertz
kHz	Kilohertz
NIHL	Noise-induced hearing loss
NIOSH	National institute of occupational safety and health
NIPTS	Noise-induced permanent threshold shift

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NU-6	Northwestern University Auditory Test No. 6
OHC	Outer hair cell
OSHA	Occupational safety and health administration
PRO	Patient reported observation
PTS	Permanent threshold shift
RSIR	Revised Speech intelligibility rating test
R-SPIN	Revised speech perception in noise test
SIR	Speech intelligibility rating test
SNR	Signal to noise ratio
SPIN	Speech perception in noise test
SPRINT	Speech recognition in noise test
sSRT	Sentence-based speech reception threshold
STS	Standard threshold shift (OSHA), significant threshold shift (NIOSH)
TFI	Tinnitus functional index
TTS	Temporary threshold shift
TTS_2	Temporary threshold shift measured 2 min after noise exposure ends
QuickSIN	Quick sentence in noise test
WIN	Words in noise test

9.1 Introduction

The role of oxidative stress in noise-induced hearing loss (NIHL) was described in detail by Altschuler (Chap. 7) and Yamashita (Chap. 8). Translational research studies assessing the prevention of acquired hearing loss in animals have been encouraging, and multiple detailed reviews of this literature are available (Abi-Hachem et al. 2010; Poirrier et al. 2010; Le Prell and Bao 2012). Data from human subjects are much more limited, but several small-scale studies suggest potential benefit from agents including magnesium (Attias et al. 1994, 2004), vitamin B_{12} (Quaranta et al. 2004), and alpha-lipoic acid (Quaranta et al. 2012). Our own research efforts, two completed clinical trials being prepared for review, have evaluated additional compounds with potential benefits against NIHL (NCT00808470; NCT01444846). A number of other agents are entering human clinical trials, and readers are encouraged to visit the National Institutes of Health online Clinical Trials Registry (http://clinicaltrials.gov/) for easy access to up-to-date clinical trial information. While we review current trials as part of this chapter, the field continues to advance at a rapid pace, and the information provided in this chapter will surely become quickly dated. In the following sections, we review several metrics and designs for human clinical trials and discuss these strategies for assessing the prevention of NIHL in humans. At the time of writing this chapter, there are no therapeutics approved by the US Food and Drug Administration (FDA) for the prevention of NIHL or hearing loss acquired secondary to other insults such as aminoglycoside antibiotic exposure or cisplatin treatment (see Chaps. 10–12). Clinical trials are an essential requisite step in the quest for approval of new agents by the FDA for the purpose of hearing loss prevention.

9.1.1 Clinical Trial Considerations

9.1.1.1 Functional Metrics

A variety of test metrics have been used to assess potential protection against NIHL by otoprotective agents. In animal models of NIHL, the evidence for efficacy is typically based on the prevention of changes in thresholds assessed using the auditory brainstem response (ABR) or distortion product otoacoustic emissions (DPOAEs) (for detailed recent review, see Le Prell and Bao 2012). Behavioral thresholds have also been used in animal studies, but these measures are less common given the time required for animal training. In contrast, for human work behavioral thresholds are considered the "gold standard," with relatively fewer studies using DPOAEs, or ABR, as the primary outcome.

Reliance on behavioral thresholds in human clinical studies is not surprising. Explicit criteria defining significant (noise-induced) threshold shift have been provided by a number of government agencies. The Occupational Safety and Health Administration (OSHA) defines a standard threshold shift (STS) as "a change in hearing threshold relative to the baseline audiogram of an average of 10 dB or more at 2,000, 3,000, and 4,000 Hz in either ear" (OSHA 1983). Employers are allowed to, but are not required to, correct annual audiograms to allow for the contribution of aging (presbyacusis) to changes in hearing level; if age correction is used, there are specific OSHA-approved tables that provide the age correction values. The National Institute of Occupational Safety and Health (NIOSH) advocates a criterion for significant threshold shift (STS) which differs from OSHA's STS. The NIOSH recommended definition of change is "an increase of 15 dB in hearing threshold level (HTL) at 500, 1000, 2000, 3000, 4000, or 6000 Hz in either ear, as determined by two consecutive audiometric tests," with the second test required to reduce false-positive findings (NIOSH 1998). The Department of Defense (DoD) defines a significant threshold shift (STS) as "a change in hearing threshold relative to the initial reference audiogram of an average of 10 dB or more at 2000, 3000, and 4000 Hz, in either ear. Age corrections will not be applied. A single frequency 15 dB shift at 1000, 2000, 3000, or 4000 Hz is considered an early warning flag with no requirements for follow-up testing or referrals, but with a requirement to counsel the patient and check hearing protection" (US Department of Defense 2004). Given these different standards, one potential strategy for assessing a protective agent is assessing whether an agent reduces the percent of workers, soldiers, or other noise-exposed participants that meet the OSHA, NIOSH, or DoD criteria for an STS. In future sections, we will refer to these criteria as "OSHA STS," "NIOSH STS," or "DoD STS" since all three agencies use the same "STS" abbreviation but have different definitions of how STS is defined.

Whereas OSHA STS, NIOSH STS, and DoD STS are all logical criteria for assessing the prevention of NIHL, there are other metrics that may be equally reasonable, including, for instance, compensable hearing loss. However, how workers compensation and disability payments are determined can be quite complicated and

inconsistent. Worker compensation laws exist in all 50 states; but, while hearing loss is a compensable injury in most states, the rules for compensation apply varying formulas in defining hearing disability. As per the excellent review by Dobie and Megerson (2000), all rules regarding compensation for NIHL have in common the use of conventional pure-tone audiometry at a subset of audiometric frequencies including 500, 1,000, 2,000, and 3,000 Hz, and sometimes, 4,000 or 6,000 Hz. Compensation for hearing loss is awarded to former military personnel, under service-connected disability, through the Veterans Administration (VA). Per 38 CFR 3.385, "impaired hearing is considered a disability for VA purposes when the auditory threshold in any of the frequencies 500, 1,000, 2,000, 3,000, 4,000 Hz is 40 dB hearing level (dB HL) or greater, the auditory thresholds for at least three of the frequencies 500, 1,000, 2,000, 3,000, or 4,000 Hz are 26 dB HL or greater, or speech recognition scores using the Maryland CNC Test are less than 94 %." Given all of the possible conventional audiometric outcomes that might be relevant when defining hearing loss for medical and legal purposes, it is not surprising that most studies have relied on a much more simple metric: average threshold shift at the frequencies most commonly affected by noise exposure, i.e., 3,000, 4,000, or 6,000 Hz (see Table 9.1 for summary of completed trials).

As summarized in Table 9.1, most studies have relied on reduced change in the amount of threshold shift at one or more frequencies across all subjects as evidence of benefit, and a smaller number of studies have based conclusions on a smaller proportion of patients/subjects exhibiting significant hearing loss. DPOAEs have been used to a lesser extent and have more commonly served as a secondary outcome. Additional tests that have not been widely used in completed trials, but might be considered for use as a measure of efficacy of novel therapeutic agents, include extended high-frequency (EHF) audiometry, speech-based audiometric tests, and measurement of the ABR. These tests are discussed in the following sections.

9.1.1.2 Distortion Product Otoacoustic Emissions

DPOAEs are well known for their use as a sensitive and objective measure of cochlear non-linear gain and an index of outer hair cell (OHC) electromotility and inner ear health (Kujawa et al. 1994; Kemp 1997; Hall 2000). Because noise principally damages OHCs, it is not surprising that DPOAEs have shown high sensitivity to noise injury in animal and human studies (Korres et al. 2009; de Souza Chelminski Barreto et al. 2011; Ramma et al. 2011; Meinke et al. 2013). DPOAE reduction is strongly correlated with threshold shift (Seixas et al. 2004, 2012; Sisto et al. 2007; Helleman et al. 2010; Müller et al. 2010), although there are some discrepancies across studies (Shupak et al. 2007; Helleman and Dreschler 2012). There are multiple suggestions that early deficits in DPOAE amplitudes indicate damage to the inner ear that precedes changes in conventional audiometry. DPOAEs have been described by multiple groups as providing predictive value for later pure-tone detection threshold deficits (Lapsley Miller et al. 2004, 2006; Job et al. 2009). Thus, several completed clinical trials seeking confirmatory evidence for protection of

Table 9.1 Completed clinical trials assessing protection against NIHL

	Population	Drug and dose	Noise	Outcome
Attias et al. (1994)	300 normal-hearing (<20 dB HL from 1 to 8 kHz) recruits completing 2 months of basic training; ages 17.7–18.5 years, all M; 255 subjects completed study	6.7 mmol (167 mg) Mg aspartate once/day every day during 2-month training period vs. placebo	M16 firearm training 6 days/week × 8 weeks; ~420 shots per person, mean peak level = 164 dBA but participants did wear ear plugs	• 28/250 Mg-treated ears had PTS > 25 dB (11.2 %); 65/260 placebo ears had PTS > 25 dB (25 %) • Bilateral PTS > 25 dB in 11.5 % of placebo vs. 1.2 % Mg • Nausea in 8 % of placebo vs. 11 % of Mg; stomachache in 9 % of placebo vs. 17 % of Mg, vomiting in 6 % of placebo vs. 3 % of Mg, diarrhea in 11 % of placebo vs. 12 % of Mg • Tinnitus in 10 % of placebo vs. 7 % of Mg; dizziness in 14 % of placebo vs. 12 % of Mg, headache in 20 % of placebo vs. 14 % of Mg
Attias et al. (2004)	20 normal-hearing (≤20 dB HL from 1 to 8 kHz) participants ages 16–37, all M; all subjects participated in untreated phase, placebo phase, and Mg phase	122 mg Mg, delivered as Mg aspartate once/day for 10 days vs. placebo; there was also an untreated control condition	White noise: 90 dB sensation level (SL) × 10 min; three exposures per subject	• Largest TTS immediately post-noise 40 dB for placebo and untreated vs. 35 dB for Mg • TTS ≥ 20 dB observed for 28 % of placebo/untreated ears vs. 12 % of Mg-treated ears (p's < 0.001) • Average TTS smaller at all frequencies from 1 to 8 kHz (all p's ≤ 0.05) • GI symptoms (nausea, stomachache, vomiting, diarrhea) in 11 % of Mg phase vs. 9 % in placebo phase • Tinnitus, dizziness, or headache symptoms in 11 % of Mg phase vs. 13 % in placebo phase
Quaranta et al. (2004)	20 normal-hearing participants (<15 dB HL), ages 20–30 years; gender not specified	Vitamin B_{12} (1 mg cyanocobalamin once daily for 7 days and 5 mg on the eighth day); there was also an untreated control condition for each subject	Narrowband (750-Hz bandwidth) noise centered at 3 kHz, 112-dB SPL, 10 min; 2 exposures per subject	• For within-subjects (untreated vs. B_{12}) comparisons, untreated TTS was reduced from 16.6 to 10.2 dB at 3 kHz and from 21.5 to 16.9 dB at 4 kHz (p's < 0.05) • For between-subjects (placebo vs. B_{12}) comparisons, TTS was significantly reduced at 3 kHz (p < 0.001) and approached significance at 4 kHz (p = 0.061)

(continued)

Table 9.1 (continued)

	Population	Drug and dose	Noise	Outcome
Kramer et al. (2006)	31 normal-hearing participants (<25 dB HL), ages 19–29 years, 14 M, 17 F	900 mg *N*-acetylcysteine (NAC) delivered as effervescent tablet 30 min prior to nightclub entry	Nightclub with music; 92.5–102.8 L_{avg} for 2 h (average exposure across 8 evenings was 98.1 dBA)	• Average TTS at 4 kHz for all subjects: 14.1 dB measured within 5 min after leaving nightclub); 9.8 dB measured ~20 min after leaving nightclub; no significant differences between NAC and placebo
				• Average DPOAE amplitude for $f_2/f_1 = 1.2$ and $L_1/L_2 = 60/50$ for f_2s = 2,3,4,5,6, and 8 kHz: most robust amplitude decrease was ~8 dB at 5 and 6 kHz; no significant differences between NAC and placebo
Suckfuell et al. (2007)	11 patients suffering from acute acoustic trauma with hearing loss of at least 30 dB at 4 and/or 6 kHz, seen within 24 h of noise exposure, 10 M, 1 F	Unilateral intratympanic injection of AM-111 at concentrations of 0.4 mg/mL ($n = 7$) or 2 mg/mL ($n = 4$) within 24 h of noise exposure, 250 µl injection followed by 30 min laying on side	Firecracker exposure on New Year's Eve	• Average PTA threshold at 4 and 6 kHz before treatment was 36 ± 16 dB
				• Primary end point was hearing threshold recovery on day 30; average PTA improvement was 11 ± 14 dB on day 30
				• Secondary end point was hearing threshold recovery on day 3; average PTA improvement was 11 ± 12 dB on day 3
				• Change in tinnitus intensity was also a secondary end point
				• Average tinnitus intensity score before treatment was 4.3 (on scale of 1–10)
				• Average tinnitus intensity score on day 3 was 4.1
				• Average tinnitus intensity score on day 30 was 3.3
				• None of the 9 patients reporting tinnitus at baseline were tinnitus-free on day 30

Fetoni et al. (2009)	20 normal-hearing participants (≤20 dB HL from 125 Hz to 8 kHz), ages 23–28 years, all M	Coenzyme Q10 terclatrate (QTer®); 200 mg once daily for 7 days vs. placebo	White noise, 90 dB HL × 15 min	• No significant differences between QTer and placebo PTA thresholds before, 1 h, or 7 days after sound exposure (p's > 0.05) • TTS was not reported • Baseline (pre-noise) PTA was not provided • Average DPOAE amplitude for $f_2/f_1 = 1.22$ equilevel (L_1 and $L_2 = 70$ dB SPL) for nine f_2s = ranging from 1,001 to 6,354 Hz • DPOAE amplitude decreases were different for QTer vs. placebo at 1 h post-noise for f_2's from 3,174 to 6,348 Hz • DPOAE amplitude decreases were different for QTer vs. placebo at 16 h post-noise for f_2's at 5,042 and 6,348 Hz • DPOAE amplitude decreases were not different for QTer vs. placebo at 7 and 21 days post-noise
Lin et al. (2010)	53 M workers employed in steel manufacturing factory for at least 1 year; average work history was 16.3 years and most workers were middle-aged	1,200 mg *N*-acetylcysteine (NAC) delivered once daily for 14 days vs. placebo (within subjects crossover design)	Daily exposure in factories ranged from 88.4 to 89.4 dBA TWA	• Mean TTS at 3, 4, and 6 kHz (HF PTA) was 2.77 dB after placebo and 2.45 after NAC ($p = 0.03$) • In a secondary analysis, the subjects that were null for both GSTM1 and GSTT1 were extracted and the effect of the drug was identified as "more prominent" ($p = 0.004$) in those 20 subjects. Such participants presumably have low glutathione S-transferase (GST) enzymatic activity • There was no reliable drug effect in the other subjects that carried the genotypes for the GSTs

(continued)

Table 9.1 (continued)

	Population	Drug and dose	Noise	Outcome
Le Prell et al. (2011)	31 participants (10 officers and 21 trainees) with average age of 25.4 years old; 27 M, 4 F	18 mg beta-carotene, 500 mg ascorbic acid (vitamin C), 305 mg alpha-tocopherol acetate (270 mg vitamin E), 1,949 mg magnesium citrate (315 mg Mg); all subjects participated in both treatment conditions (within subject crossover design)	Two rounds of ammunition (20 shots per round) fired from an automatic machine gun (Ksp-58) inside a bunker over a period of less than 1 min	• No reliable effect of shooting exercises on hearing thresholds in either placebo or nutrient condition using single-frequency data or maximum shift at 3, 4, or 6 kHz in either ear • The two subjects with > 10 dB TTS in the placebo condition had 12-dB less TTS in the treatment condition • Three of the four subjects with 8 dB TTS in the placebo condition had 2–4 dB less TTS in the treatment condition; the fourth subject with 8 dB TTS in the placebo condition had 8 dB TTS in the treatment condition as well
Lindblad et al. (2011)	23 untreated participants and 11 treated participants, 6 of the untreated subjects also participated in the treatment condition; ages 22–50 years; 42 M, 2 F	200 mg acetylcysteine (NAC) immediately after exposure, 1 h postexposure, the following morning at breakfast, and 1 h later	Two rounds of ammunition (20 shots per round) fired from an automatic machine gun (Ksp-58) inside a bunker over a period of less than 2 min; sound levels were 164–166 dB SPL 2 m from the weapon and 135–154 in the ear canal under the hearing protectors	• No reliable effect of shooting exercises on hearing thresholds in untreated participants or NAC-treated participants • Left ear thresholds more variable than right ear thresholds in untreated participants at 3 and 4 kHz; no statistically significant effect of NAC on observed variability • Psychoacoustic modulation transfer function was decreased in control subjects post-shooting, with fewer observed changes in NAC-treated subjects

Quaranta et al. (2012)	30 normal-hearing participants (<20 dB HL from 500 Hz to 8 kHz), ages 20–30 years, 15 M, 15 F	600 mg alpha-lipoic acid delivered once 1 h prior to noise or once daily for 10 days vs. untreated controls	3-kHz pure tone at 90-dB HL for 10 min, unilateral	• TTS 2-min post noise was significantly reduced at 6 kHz in the 10-day pretreatment group (TTS = 7.3 ± 7.6 dB) relative to untreated subjects (TTS = 15.6 ± 7.3 dB, $p = 0.023$) and single-dose subjects (TTS = 14.1 ± 5.6 dB, $p = 0.035$), with no differences at 3 or 4 kHz. • TEOAE change was different in 10-day pretreatment group (−0.2 ± 0.96 dB), untreated group (0.7 ± 1.17 dB), and single-dose group (1.0 ± 1.0 dB) (ANOVA $p = 0.0278$)
Kopke et al. (2015)	566 normal hearing participants (≤25 dB HL at 2, 3, 4, and 6 kHz)	900 mg N-acetylcysteine (NAC) tablets, t.i.d., total of 2700 mg/day during first 13 days of weapons training, followed by b.i.d. dosing for 3 days, total of 1800 mg/day	M16 weapons training (325 rounds) and other noise, including simulated explosions	• STS defined as an increase of 20 dB or more at one frequency or 10 dB or more at two adjacent frequencies was not reliably different for the left ear (NAC: 21 % vs Placebo: 19 %, $p = 0.7816$) but approached $p < 0.05$ for the right ear (NAC: 21 % vs Placebo: 27 %, $p = 0.0562$) • STS defined as an increase of 15 dB or more at one frequency or 10 dB or more at two adjacent frequencies was reliably different in the trigger-hand ear (NAC: 27 % vs Placebo: 35 %, $p = 0.0288$)

the human inner ear have already included DPOAE tests as a metric of potential protection against noise-induced cell damage. Currently, changes in DPOAE amplitude are the most common secondary outcome for noise-induced change in function in clinical trials assessing otoprotection (Attias et al. 2004; Kopke et al. 2015; Kramer et al. 2006; Le Prell et al. 2011).

Challenges in implementing the use of DPOAEs in clinical decision-making, specifically in the context of NIHL, are the topic of a recent paper by Konrad-Martin et al. (2012b). We suggest here that DPOAE-related protection would be an encouraging positive outcome; however, protection measured using DPOAE metrics should be interpreted with caution. If protection of DPOAE responses is ultimately shown to reduce later deficits during conventional pure-tone audiometry, then DPOAE data will become more useful for identifying benefits of novel drugs. Alternatively, because DPOAEs fail to identify selective damage to IHCs and the auditory nerve, the widespread use of DPOAEs may be more limited. It is interesting that Avan and Bonfils (2005) described a subgroup of workers with NIHL, defined by a change in behavioral hearing sensitivity, without compromised DPOAE functions, suggesting potential inner hair cell or neural involvement.

9.1.1.3 EHF Audiometry

EHF audiometry is used to assess pure-tone thresholds at higher frequencies than are typically monitored in a hearing conservation program or during traditional audiometric monitoring; the EHF frequency range is defined as extending from 9 to 20 kHz. Elevated EHF thresholds have been observed in individuals with a history of noise exposure (Vassallo et al. 1968; Osterhammel 1979; Erickson et al. 1980; Fausti et al. 1981a, b). Because significant EHF deficits have also been reported in patients treated with ototoxic drugs such as the antineoplastic drug cisplatin (Fausti et al. 1984b; Tange et al. 1985) and aminoglycoside antibiotics (Fausti et al. 1984a), EHF testing has been proposed for use in identifying early changes in hearing following either physical trauma (i.e., noise) or pharmacological insult. Longitudinal studies assessing EHF thresholds as a function of noise are limited. In a single longitudinal study that followed a sample of 14-year-old students over a 3-year period, the largest threshold changes were observed at the two highest frequencies tested (14 and 16 kHz) with changes being approximately 5 dB (Serra et al. 2005). The observed EHF changes were attributed to concert and discothèque attendance (Biassoni et al. 2005).

In contrast to longitudinal studies, cross-sectional studies are more common. Our own data, as well as those from other labs, also suggest potential EHF loss with long-term use of personal music player devices, although the deficits tend to be limited (Meyer-Bisch 1996; Peng et al. 2007; Kim et al. 2009; Figueiredo et al. 2011; Le Prell et al. 2013), and some groups report no threshold deficits (Wong et al. 1990; Mostafapour et al. 1998; Kumar et al. 2009; Shah et al. 2009). Because none of those data are longitudinal, it remains possible that other unreported noise exposures contributed to the reported group differences. The most robust support for noise-induced deficits at EHF frequencies comes from a cross-sectional analysis

of hearing thresholds in adult male factory workers, with varied noise exposure (Ahmed et al. 2001). In a group of workers with thresholds that were 20-dB HL or better from 250 Hz to 8 kHz, the subset of workers that were routinely exposed to noise had deficits at frequencies from 12 to 20 kHz, whereas workers that were not assigned to noisy areas did not have these EHF threshold deficits. Those data have been interpreted to suggest that EHF threshold deficits precede hearing loss at lower frequencies after noise, as observed following ototoxic drug therapies. Changes at EHF frequencies may ultimately prove to be a useful tool for identifying individuals with increased vulnerability to noise insult (see Osterhammel 1979) or those likely to develop NIHL at conventional test frequencies (250 Hz to 8 kHz). However, longitudinal studies, incorporating serial monitoring, are essential for determining whether workers with EHF deficits go on to develop hearing loss at lower frequencies over time.

If EHF tests provide a reliable sensitive metric for early effects of noise on the inner ear, then EHF testing might have some utility for hearing conservation monitoring. However, we are reluctant to advocate EHF testing for use as a primary outcome in clinical trials assessing otoprotective agents based on a variety of studies in which noise-induced EHF deficits have not been reliably observed. For example, when 18–21-year-old male soldiers not yet exposed to military weapons noise were compared to young soldiers seen after acute acoustic trauma, there were no threshold differences from 12.5 to 20 kHz; the deficits in the noise-exposed personnel were greatest at 4–8 kHz and extended only to 11.2 kHz (Balatsouras et al. 2005). When conventional and EHF thresholds measured in Finnish Air Force Military Personnel (19–48 years old; 50 male, 1 female) were compared to Finnish normative data, there were no hearing deficits for either conventional or EHF stimuli (Kuronen et al. 2003). Data from musicians are mixed. Minimal EHF threshold deficits (at 12.5 and 14 kHz) in conjunction with deficits at conventional test frequencies (3–8 kHz) were present in one group of musicians (Schmuziger et al. 2006), and EHF deficits developed slowly in a group of swedish pop musicians followed over time (Axelsson and Lindgren 1978; Axelsson et al. 1995), but deficits were absent in other groups (Johnson et al. 1985, 1986).

EHF deficits have not been any more reliable in temporary threshold shift (TTS) studies. Comparison of pre- and postflight hearing tests revealed small but statistically significant TTS at both conventional and EHF frequencies (approximately 1–3 dB) in military personnel, suggesting no additional benefit was obtained by supplementing a conventional hearing test paradigm with EHF testing (Kuronen et al. 2003). In a study on TTS after music player use, EHF deficits did not accompany TTS measured at lower frequencies (Le Prell et al. 2012). When TTS after music rehearsal was evaluated, TTS was detected at frequencies at and below 8 kHz but not at or above 9 kHz (Schmuziger et al. 2007). In summary, current clinical and industrial practice does not include routine monitoring for NIHL at frequencies beyond 8 kHz, and EHF testing has not reliably produced significant sensitivity above conventional testing. Thus, if otoprotective efficacy is limited to EHF frequencies, protection should be interpreted with caution until reduction in EHF deficits can be shown to predict hearing retention at conventional frequencies.

9.1.1.4 Speech-Based Functional Tests

The use of word recognition, and more specifically, words or speech-in-noise tests, may be useful in otoprotection studies, as it has long been shown that the typical listener with high-frequency hearing loss, including noise-induced, has disproportionate difficulty in noise (Quist-Hanssen et al. 1979). The American Academy of Otolaryngology (AAO) has recently recommended that word recognition scores should be included in *all* clinical trials that assess auditory function (Gurgel et al. 2012). To the best of our knowledge, speech-based functional tasks have not been used in any of the completed clinical trials assessing otoprotective agents. Interest and enthusiasm for these tasks appear to be increasing given the repeated suggestions that loss of neural connections from inner hair cells that result in decreased ABR amplitude *in the absence of overt permanent threshold shift* may also result in poorer speech-in-noise discrimination (Kujawa and Liberman 2009, 2015; Lin et al. 2011; Makary et al. 2011). Given these recent recommendations, several tests that might be considered for use in clinical trials are discussed below.

The following section focuses on speech-in-noise tests rather than speech-in-quiet tests based on recommendations that speech-in-noise tests be considered a "stress test" for auditory function (Wilson 2011). As per the review by Wilson, it was Carhart (1951) who first recognized that the ability to understand speech in noise was an important measure of function as some patients reported disproportionate difficulty hearing in noise. Later, Carhart and Tillman (1970) explicitly advocated that audiologic evaluations should include a measure of the ability of patients to understand speech in competing background noise in addition to the traditional pure-tone audiogram and speech discrimination in quiet.

Following the suggestion by Carhart and Tillman (1970), new tests began to emerge, including, for example, the Speech Perception in Noise (SPIN) Test (Kalikow et al. 1977). The test items in the SPIN test are sentences read by a male talker presented with a simultaneous 12-voice multi-talker babble, and the listener's task is to identify the last word in the sentence, which is always a monosyllabic noun. The SPIN test has a total of 250 words divided across ten lists, with each list containing 12–13 alternate types of sentences, including a low-predictability condition where the final word is difficult to predict based on sentence context and a high-predictability condition where the final key word is somewhat predictable using sentence context. The background babble is presented at the same level as the sentences in the original description (Kalikow et al. 1977). Later revisions of the test (Revised Spin Test, R-SPIN) implemented a protocol with the speech track presentation level set at 50 dB above the estimated threshold and the signal-to-babble ratio set at 8 dB (Bilger et al. 1984). The R-SPIN was composed of only 200 target words, with the target words divided into four list pairs, each consisting of two 50-sentence lists. The paired lists had the same words, presented in the opposite context conditions (high context or low context). One of the important outcomes from a study by Bilger et al. (1984) was the finding that the ten different word lists were not equivalent, with large differences in mean performance on the low-context sentences within each of the ten word lists suggesting the different lists could not be used interchangeably.

The Connected Speech Test (CST) emerged shortly after the R-SPIN (Cox et al. 1987, 1988). Intended primarily to quantify hearing aid benefit, the test was composed of sentences spoken by a female speaker presented at six signal-to-babble ratios ranging from −3 to −8 dB. The original set of 72 sentences was ultimately reduced to 48 test sentences and 9 practice sentences, with all sentences composed of basic vocabulary and syntactically simple sentences, and the scoring criteria selected such that most normal-hearing listeners achieved test scores of approximately 50 % correct (Cox et al. 1987). The lists were divided into two sets of 24 sentences to facilitate test–retest comparisons. When the test was applied to listeners with hearing loss, there were a subset of patients that had disproportionate difficulty in noise (Cox et al. 1988). Several other tests followed, such as the Speech Intelligibility Rating (SIR) test (McDaniel and Cox 1992; Beck and Speaks 1993) and the Revised Speech Intelligibility Rating (RSIR) test (Speaks et al. 1994).

The Hearing in Noise Test (HINT) was the next to emerge (Nilsson et al. 1994). During the development of the HINT, the Bamford–Kowal–Bench (BKB) sentences described by Bench et al. (1979) were modified for American English, as the British words and sentences were not commonplace for American listeners. The HINT includes a large set of sentence materials (250 sentences, spoken by a male speaker) with the sentences selected to have relatively uniform length. The sentences are divided into 25 phonemically balanced lists, with ten sentences per list, used for measuring sentence-based speech reception thresholds (sSRTs). Sentences are presented both in quiet and in spectrally matched masking noise. The HINT remains one of the more common tests used clinically and across research studies. Another relatively well-defined test is the Quick Speech-In-Noise (QuickSIN) test (Killion et al. 2004). The QuickSIN is a nonadaptive test of speech perception in four-talker babble. The test consists of sentence lists (six sentences per list) with five target words per sentence (yielding 30 target words per list). Sentences are syntactically correct but contain few semantic or contextual clues. Sentences are presented at a fixed level, and the SNR is sequentially decreased. Participants repeat the sentences and their SNR loss is calculated based on the number of target words correctly recalled. Further details are available in the QuickSIN User's Manual. There is an anecdotal report that patients believe the QuickSIN better estimates their actual difficulty with listening in noise (Anderson et al. 2012).

The Words-in-Noise (WIN) test is perhaps now among the best developed tests; the WIN test measures word recognition performance for words presented in multi-talker babble at seven signal-to-noise ratios with the 50 % correct point (in dB SNR) used as the primary performance metric (for review of data across studies, see Wilson 2011). In brief, the WIN was initially designed as a 70-word instrument; there were ten unique words to be presented at seven signal-to-noise (S/N) ratios decreasing from 24 (easiest) to 0 (most difficult) in 4-dB decrements (Wilson et al. 2003). This 70-word list was subsequently divided into two 35-word lists with equivalent recognition performance established for the two lists (Wilson and Burks 2005). A third list (WIN List 3) has been developed for use as a practice list to familiarize participants with the task of listening to words presented in background babble (Wilson and Watts 2012). The multi-talker babble has six female voices. The WIN has been validated against the Speech Recognition in Noise Test (SPRINT)

(Wilson and Cates 2008). We note here that the SPRINT was developed by and is used by the US Army to assess communication ability and fitness for duty. As described by Wilson and Cates (2008), the WIN and the SPRINT are similar in that both use Northwestern University Auditory Test No. 6 (NU-6) words. They differ in that the WIN uses a smaller number of words and a larger number of signal-to-noise ratios (S/N). Whereas the WIN has seven discrete S/N rations in 4-dB steps of increasing difficulty (Wilson and McArdle 2007; Wilson and Cates 2008), the SPRINT includes multi-talker background babble presented at a single (9 dB) S/N ratio. Directions (written by Cord et al., undated) and sound files are available online at http://militaryaudiology.org/site/2009/01/sprint-test/.

In summary, the early observations by Carhart (1951) that some patients have disproportionate difficulty in noise has been repeatedly confirmed, with multiple investigators reporting that participants with similar audiometric thresholds vary significantly with respect to speech recognition performance in background noise (Cox et al. 1988; Smoorenburg 1992; Vermiglio et al. 2012; Grant et al. 2013). Vermiglio et al. (2012) reported that the ability to recognize speech in steady-state noise could not be predicted from the audiogram and suggested a new classification scheme for hearing impairment requires both the audiogram and speech-in-noise thresholds. It is highly relevant that there are now proposals that the HINT be used as a diagnostic tool for employment decisions for jobs where communication performance is critical (Giguère et al. 2008). It is difficult to provide good guidance on the selection of speech-in-noise tasks for clinical trials as there have not been many studies that provide empirical data directly comparing performance of participants across speech-in-noise tests. There are some notable examples of studies that do offer back-to-back comparisons, such as Wilson et al. (2007) and Grant and Walden (2013), and based on these data, we suggest that either the WIN or the QuickSIN would appear to be reasonable choices.

9.1.1.5 Auditory Brain Stem Response

Interest in ABR tests as a primary metric in human research has been increasing given that several groups have reported loss of neural connections from inner hair cells, with corresponding decrease in ABR amplitude, after noise exposures that produce a robust threshold shift (i.e., approximately 40-dB TTS, measured 24-h post-noise) (Kujawa and Liberman 2009; Lin et al. 2011; Wang and Ren 2012). Although primary spiral ganglion degeneration, in the absence of sensory cell loss, has been reported in human temporal bones, noise history was only known for three ears (Makary et al. 2011). Physiological data from humans supportive of these claims are also limited. When ABR amplitude and latency data from a large population of veterans with known noise exposure were compared to data collected in other nonveteran populations, there were no qualitative differences (Konrad-Martin et al. 2012a). The effects of noise were explored as a secondary analysis, however, with age effects being the primary variable of interest. When the potential for

noise-induced ABR deficits was specifically assessed in professional pop/rock musicians, no deficits in ABR amplitude were detected (Samelli et al. 2012). However, that study only included a limited number of subjects (16 musicians and 16 controls) over a broad range of ages (21–43 years old). ABR input–output functions are a "gold standard" metric for assessing lasting effects of noise on the neural population. However, there have not been any large-scale studies that evaluate whether behaviors that result in TTS are correlated with reduced suprathreshold ABR amplitude in humans. If human ABR amplitude varies with noise history, in the absence of threshold deficits, we may ultimately gain insight into the "critical boundary" at which noise becomes hazardous to synapses in human ears, a boundary that is not currently known for either humans or laboratory animals (for discussion of critical boundary, see Le Prell et al. 2012; Spankovich et al. 2013). For the purpose of clinical trial guidance, we note here that the sensitivity of ABR recordings is improved using electrodes that utilize the ear canal as a recording site (Gaddam and Ferraro 2008); the amplitude of ABR Wave I was significantly larger and easier to identify when the ear canal was used as one of the recording sites in comparison to more conventional scalp (mastoid) recordings. Amplitude of Wave I may not be the only evoked potential of interest. Recently, Ruggles et al. (2011, 2012) identified variability in the auditory brainstem frequency-following response in normal-hearing young adults as a key factor for difficulty discriminating sounds in noise. Other groups are independently arriving at similar conclusions; Hopkins and Moore (2011) also reported that reduced sensitivity to temporal fine structure cues may underlie speech perception difficulties. Stamper and Johnson (2015) reported smaller ABR Wave I amplitudes in humans for data collected using mastoid electrodes, but not tympanic membrane electrodes, when amplitude was assessed as a function of noise history. Unfortunately the authors did not assess the potential role of gender, which is an important confound. Males typically have smaller ABR amplitudes and longer ABR latencies than females (see Hall 1992). If they also report more sources of noise exposure, the purported effect of noise may be related to gender, rather than noise history.

9.1.1.6 Tinnitus

Patient-reported observations (PRO) are the standard for determining the incidence, loudness, and severity of subjective tinnitus. A visual analogue Tinnitus Loudness Rating Scale has been used to determine the incidence, loudness, and severity of subjective tinnitus reported by study subjects (as used in NCT00808470; Le Prell et al. 2012; Spankovich et al. 2013). At study onset, participants complete a brief questionnaire to determine the extent to which they normally experience tinnitus in either or both ears, with tinnitus operationally defined as a hissing, ringing, or other auditory sensation in the absence of an external sound. For clinical trials, we would advocate that participants indicating that tinnitus is experienced frequently (at least one incidence daily) or with significant severity (defined as severe enough to

distract their attention from and/or disrupt ongoing activity) be asked to complete the Tinnitus Functional Index (TFI). The TFI is the most recent tinnitus-related screening tool; it has been responsive to treatment effects, and data suggest a 13-pt change is clinically significant (Meikle et al. 2012). Other well-validated scales include the Tinnitus Handicap Questionnaire (Kuk et al. 1990), the Tinnitus Reaction Questionnaire (Wilson et al. 1991), and the Tinnitus Severity Scale (Sweetow and Levy 1990). Each has been well-validated for use in patients who experience severe tinnitus. However, it is difficult to predict whether these questionnaires will be sensitive to subtle changes in the frequency or severity of tinnitus associated with study participation, as might be predicted with long-term daily exposure to occupational noise. Moreover, it is not clear which of these scales, if any, will be sensitive to changes in noise-induced tinnitus percepts in clinical trials. Participants reporting tinnitus at the time of audiometric testing should be asked to participate in pitch-matching and loudness-matching tasks to more precisely characterize the nature of the noise-induced tinnitus if possible.

9.1.1.7 Summary of Clinical Trial Metrics

Studies assessing otoprotective benefits in both animals and humans have primarily focused on the ability of these agents to reduce or prevent threshold shift. However, there is an emerging body of evidence that suggests that in addition to threshold shift or lack thereof, there may be important changes in hearing at suprathreshold levels. Provocative data from animal studies suggest that some noise exposures that do not result in threshold shift can show evidence of damage to synapses on auditory nerve fibers. Suprathreshold deficits including speech discrimination and speech discrimination in noise are common, but there is little agreement whether to use the WIN, QuickSIN, HINT, SPRINT, or other tests. Noise-induced ABR amplitude changes are a "hot topic" in animal models, but such changes have not been established in humans. Data collection paradigms in use in recently completed studies that are not yet described in the literature and other ongoing clinical trials on the prevention of NIHL are summarized in Table 9.2.

9.1.2 Clinical Trial Paradigms

The Committee on Hearing, Bioacoustics, and Biomechanics (CHABA) has proposed a model where the risk of PTS is assumed to vary as a function of measured TTS deficits. The CHABA group specifically postulated that TTS measured 2 min after an 8-h work noise exposure ends (TTS_2) is a measure "that will correlate with the ability of a single-day's exposure to produce a noise-induced,

permanent threshold shift (NIPTS), if it is repeated on a near-daily basis, over a course of about 10 years" (Kryter et al. 1966). From this assertion, it could be inferred that risk of later PTS will be reduced if the daily, repeated TTS_2 is reduced or prevented. The assumption that daily TTS can be used to predict PTS has not been empirically validated; however, the CHABA criteria have not been adopted. Taken together, it is difficult to draw any real conclusions on the long-term prevention of PTS, based solely on observed reductions in daily TTS. That said, TTS in military personnel and workers in loud high-risk worksites has significant real-world impact with respect to effectiveness, injury, lethality, and survivability.

Any discussion of the ethics of NIHL otoprotection trials would be incomplete without acknowledgment of the potential for harm after noise exposures that induce robust TTS. These issues are discussed in detail in Le Prell et al. (2012), where a new laboratory-based TTS model is proposed for use in human clinical trials seeking initial "proof-of-concept" data. In brief, recent animal model data from Kujawa and her colleagues clearly show that robust TTS (40–50 dB TTS measured 24 h post-noise, in their studies) is harmful to the auditory nerve population at short post-noise intervals (in the form of decreased synaptic ribbon number and decreased amplitude of the sound-evoked auditory brainstem response; see Kujawa and Liberman 2009; Lin et al. 2011). Slow-to-develop loss of the spiral ganglion fiber population and an increase in the changes in hearing with aging were also observed over the course of the mouse life span, after exposures that induced robust (40 or more dB) TTS (Kujawa and Liberman 2006). These data contradict a long-standing assumption that, as long as thresholds recover in the short post-noise time frame, the ear itself completely recovers. These reports have raised important questions, such as whether a more moderate TTS could also induce long-term deficits through similar neural-based mechanisms. The "critical boundary" at which long-term synaptic deficits are first induced is not known; however, mice (Le Prell et al. 2015) and rats (Lobarinas et al. 2015) that had developed TTS's of 20–30 dB 24 h after the noise exposure had no decrease in ABR amplitude. Importantly, rats with larger (40 dB) TTS resulting in ABR Wave I amplitude decreases had poorer post-noise performance on a signal in noise. Such data raise important questions about the relationship among noise exposure, TTS, and deficits in speech in noise performance. It has been suggested that reduced numbers of neurons will have the additional result of increasing the difficulty of performing tasks that require discrimination of speech and other sounds in noise (Kujawa and Liberman 2009). The clinical significance of this assumption has not been empirically evaluated, and there are no regulatory requirements for testing speech in noise. Moreover, there is no disability compensation for functional difficulties with these more challenging tasks. The point here is not to suggest that such difficulties are not important, but rather, to highlight the potential challenges with respect to interpreting how scientific data may be potentially relevant to human patients. Clearly as well, these issues define specific future research targets of opportunities (for recent discussion, Kujawa and Liberman 2015).

9.1.3 Temporary Threshold Shift

9.1.3.1 Controlled Noise Models

Several laboratory-based human studies using controlled noise have induced reliable TTS and demonstrated statistically reliable reductions in TTS in subjects treated with proposed otoprotective agents. We have suggested that TTS models are appealing for initial studies given that trial duration and cost are reduced relative to the longer-duration trials needed to assess PTS. With reduced participation time for any given subject, subject attrition and loss to follow-up are less likely to be issues as well (Le Prell et al. 2012; Spankovich et al. 2013). Reduced TTS after laboratory-based noise exposure has been shown in several studies listed in Table 9.1. These studies have used broadband (white) noise, narrowband noise centered at 3-kHz, or 3-kHz pure-tone stimuli as the acoustic insult. Taken together, these paradigms have shown significant utility and success in probing otoprotective benefit. However, listening to these types of continuous signals (i.e., pure tones, Gaussian noise, shaped noise) would likely be considered unpleasant by many study participants. More importantly, real-world noise in both occupational and recreational settings is typically more variable, reflecting a mix of background noise and impulses and/or impacts (Erdreich 1986). This variation in spectral amplitude likely influences the effects of noise on the inner ear, as noise that has more impulsive components and peaks can be more hazardous than steady-state noise (Hamernik et al. 2007; Zhao et al. 2010; Goley et al. 2011). As a result, more recent studies have shifted to use more real-world noise insults.

9.1.3.2 Recreational Noise Models

Recreational noise models are appealing for clinical trials in that the subjects are exposed to loud sound in real-world, clinically relevant, situations that they would typically experience, regardless of study enrollment. Unfortunately, in the real world, noise can vary from setting to setting and even from day to day within a fixed setting. In a clinical trial using real-world nightclub noise as an insult and *N*-acetylcysteine (NAC, 900 mg) as a potential protective agent, there was up to 10-dB difference in exposure level across the subject cohorts tested on different days (with L_{avg} ranging from 92.5 dBA to 102.8 dBA across nights) (see Table 9.1); this variability in exposures across nights made it difficult to assess efficacy of the intervention in the 32 enrolled subjects (see Kramer et al. 2006). Recreational noise models remain of interest; a similar approach is being used in NCT01727492 as per Table 9.2. The study being performed at Antwerp University Hospital in Belgium is a randomized double-blind, placebo-controlled, crossover study assessing the potential prevention of NIHL and tinnitus using a combination of 200 mg magnesium and 600 mg NAC taken 1 h before leisure noise, in participants who will be exposed to noise "above 100 dB for at least 30 min" on four occasions (NCT01727492). Participants consume placebo prior to two of the recreational

Table 9.2 Current clinical trials assessing protection against NIHL

	Population	Drug, dose, design	Noise	Stated primary outcome
NCT00808470; completed	Healthy normal hearing ages 18–31 years, recruited out of the University of Florida; completed in December 2013	Once daily dietary supplement composed of 500 mg vitamin C (magnesium ascorbate), 315 mg magnesium (magnesium citrate, magnesium ascorbate, magnesium stearate), 267 mg vitamin E (d-α-tocopherol acetate), and 18 mg beta-carotene, for 4 days (3 days prior to music, on day of music); randomized, double-blind, placebo-controlled clinical trial	Rock or pop music presented at approximately 100 dBA in ear sound level	• Primary outcome: average threshold shift at 4 kHz in both ears at 15-min post-music; repeated measures at hourly intervals for 3 h; additional tests at 1 day and 1 week post-music • Secondary outcome: threshold shift at individual frequencies • Secondary outcome: DPOAE amplitude • Secondary outcome: DPOAE amplitude • Secondary outcome: tinnitus
NCT01444846; completed	Healthy normal hearing ages 18–31 years, recruited out of the University of Florida; completed in November 2013	Twice daily oral SPI-1005; 200, 400, or 600 mg for 4 days (2 days prior to music, on day of music, 1-day post-music); randomized, double-blind, placebo-controlled clinical trial	Rock or pop music presented at approximately 100 dBA in ear sound level	• Primary outcome: reduction in Temporary Threshold Shift (postexposure pure-tone audiometry)
NCT02257983; currently recruiting subjects	Healthy normal hearing ages 18–31 years, recruited out of University of Florida	Three times daily oral EPI743 (Vinceriquinone™); 400 mg for 9 days (7 days prior to music, on day of music, 1-day post-music); randomized, double-blind, placebo-controlled clinical trial	Pop music presented at approximately 100 dBA in ear sound level	• Primary Outcome: Reduction in Temporary Threshold Shift (post-exposure pure tone audiometry) • Secondary Outcome: Time to Recovery (post-exposure pure tone audiometry)
NCT01345474; currently recruiting subjects	Drill Sergeant (DS) instructor trainees, ages 21–40 years, recruited out of Fort Jackson, South Carolina, USA	Twice daily oral D-methionine of up to 100 mg/kg/day for 18 days (starting three days prior to training, during 11 days of training, and continuing four days post-training); randomized, double-blind, placebo-controlled clinical trial	M-16 weapons training (500 round minimum over 11-day period)	• Primary outcome: pure-tone air conduction threshold 22 days after cessation of weapons training, including change from baseline as measured by absolute change and frequency of STS • Secondary outcome: tinnitus scales 22 days after cessation of weapons training including change from baseline and scores for both loudness and annoyance

(continued)

Table 9.2 (continued)

NCT01727492; currently recruiting subjects	Age 18–25 years, recruited out of Antwerp University Hospital in Belgium	200 mg magnesium and 600 mg NAC taken 1 h before leisure noise; randomized double-blind, placebo-controlled, crossover study. Participants consume placebo prior to two of the recreational noise exposures and antioxidants prior to the other two recreational noise exposures	Leisure noise "above 100 dB for at least 30 min" on four occasions	• Primary outcome: protection against noise-induced tinnitus • Secondary outcome: change in tinnitus duration • Other outcome measure: decrease of temporary threshold shift; specifically, "A limited number of participants will have audiological testing (audiometry including high-frequency audiometry, speech-in-noise testing, and otoacoustic emissions) prior to the musical event as well as the morning after noise exposure in order to measure the effects of antioxidants on the hearing thresholds and hearing performance"
NCT02049073; not yet open for participant recruitment	Healthy normal hearing ages 18–30 years, to be recruited out of Washington University	Methylprednisolone 32 mg or 64 mg administered orally once on day of music exposure; zonisamide 100 mg or 200 mg administered orally once daily for 2 weeks prior to music; randomized, double-blind, placebo-controlled clinical trial	Rock or pop music presented at approximately 100 dBA in ear sound level	• Primary outcome: pure-tone thresholds at 3, 4, and 6 kHz in both ears at 15-min post-music • Secondary outcome: pure-tone thresholds at hourly intervals for 3 h • Secondary outcome: DPOAE amplitude • Secondary outcome: tinnitus handicap inventory • Other outcome measures: pure-tone thresholds at 1-week post-music

All information is current as of the writing of this chapter and as of the dates on which the trial information was verified in the clinical.trials.gov website

noise exposures and antioxidants prior to the other two recreational noise exposures (NCT01727492).

Music-player models are a subset of recreational noise models. There is a significant literature on the change in pure-tone detection thresholds or other metrics, such as DPOAE amplitude, in humans after the use of a music player. In a number of early studies, designed to identify effects of music player use on participant hearing, participants were asked to select their own listening level. This resulted in significant variability in user-selected listening levels, small sample sizes for any given listening level, and typically, TTS was measured in only the subset of the participants who chose the highest listening levels (Lee et al. 1985; Pugsley et al. 1993; Hellstrom et al. 1998). In several more recent studies, sound levels were set by the investigator, resulting in more consistent exposures across subjects, although there were still few reliable changes in function given the conservative nature of the exposure (Krishnamurti and Grandjean 2003; Bhagat and Davis 2008; Keppler et al. 2010). Our research efforts have built on this paradigm by systematically increasing listening levels until a small but reliable TTS was obtained, in order to develop a laboratory-based paradigm for use assessing potential otoprotective agents (Le Prell et al. 2012). A listening level of ~100 dBA in ear level for 4 h was identified as an appropriate intensity for future investigations assessing otoprotection. This paradigm has now been used in two recently completed clinical trials (NCT00808470; NCT01444846) and has been adopted for use by others (NCT02049073). In the two clinical trials completed at the University of Florida, we partnered with Hearing Health Science Inc. to assess a dietary supplement and with Sound Pharmaceuticals Inc. to assess a novel compound. Both studies are listed in Table 9.2. Recently, a study assessing EPI743 (Vinceriquinone™) was initiated at the University of Florida (NCT02257983).

9.1.3.3 Factory Noise Models

Like recreational noise models, factory models are appealing trial environments because the occupational noise exposure is a real-world and clinically relevant situation. A single study using this model was completed (see Table 9.1), and TTS was small, averaging less than 3 dB in both treatment conditions. Because hearing loss is a well-known problem for workers exposed to noise in factories, simulating factory noise provides compelling opportunities for the prevention of PTS that accrues slowly over time (see Sect. 9.1.4).

9.1.3.4 Impulse Noise Models

Impulse noise is distinct from continuous noise and potentially more relevant for certain military and industrial populations. The need for a laboratory-based impulse noise paradigm was made obvious to us in the course of completing a clinical trial in partnership with the Swedish military. TTS after mandatory weapons training

exercises, despite the required use of hearing protection devices, was deemed a problem, and two separate clinical trials were initiated. In two separate trials (Le Prell et al. 2011; Lindblad et al. 2011), there was a failure to measure reliable changes in hearing in both control subjects and study participants receiving active agents, which was likely a consequence of more effective earplug/earmuff use during the exercises (Le Prell et al. 2011). Increased attention to hearing conservation as part of enrollment in a study assessing hearing is consistent with the well-known Hawthorne effect. While it was positive to see that the hearing protection devices provided adequate hearing protection when used carefully and consistently, this model was not useful for the assessment of otoprotective agents in either investigation. We therefore initiated the process of developing a laboratory-based model for inducing a small but transient change in hearing using impulse noise and are progressing in the development of this model (Spankovich et al. 2013).

9.1.4 Permanent Threshold Shift

9.1.4.1 The Ethics of PTS Trials

There is a significant ethical challenge in designing and conducting human trials measuring protection against NIHL. Research investigations, including clinical trials, cannot put subjects at increased risk of PTS or other permanent damage to the inner ear in the course of a drug trial. One strategy has been to draw upon military populations that undergo weapon training that exceeds the limits of conventional hearing protection. Studies that draw on military personnel working in jobs that are likely to result in NIHL despite the use of hearing protection pose fewer ethical challenges. All participants in such studies are guaranteed protection using the same traditional mechanical devices (ear plugs, ear muffs) available to anyone NOT participating in the study. The benefit is that participants in the research study have the potential for added protection via novel therapeutic treatments if they are assigned to the treatment condition and if the treatment is in fact effective. This strategy has been used in one completed study (see Kopke et al. 2015), and a recently initiated study follows this model as well (NCT01345474; PI Kathleen Campbell). One significant limitation, however, is that access to military populations during and after weapons training is difficult to negotiate, and such populations are rarely able to avoid other non-study noise insults during the post-weapons training interval that precedes the final study hearing test. In other words, subjects may be exposed to additional harmful noise exposure after the study treatment has ended but before the final study outcomes are collected.

Identification of specific military personnel at risk for NIHL is a major challenge. While hearing loss and tinnitus are the two most prevalent disabilities for current US military personnel, there is little systematic data regarding specific population subsets that can be enrolled in clinical trials on the prevention of NIHL. The new Department of Defense Hearing Center of Excellence (HCoE) has taken an interest in this issue, and is working to bring together clinicians, scientists, and military

personnel together to begin a dialogue. As an alternative to military-based studies, some investigators have sought workforce populations required to use hearing protection but who nonetheless develop NIHL over time (based on historic documentation). These studies are also challenging, in that recreational (non-workplace) noise may vary considerably across workers that volunteer for such studies, and the extent to which workers correctly and consistently use HPDs throughout the study period may also vary. Given the slowly accruing nature of small NIHL deficits, factors that introduce variability across subjects are a major challenge.

9.2 Summary and Conclusions

This chapter has not sought to advocate for any single therapeutic approach; there are no adequate data yet available for any single agent, or combination of agents, to recommend the use of a given dose or a given product. This chapter has put forth specific criteria against which efficacy might be assessed, and the reader is encouraged to seek evidence that any therapeutic, available over the counter or by prescription, meets these criteria. As eloquently stated by W. Edwards Deming, "In God we trust; all others must bring data," (Davenport and Harris 2007).

Despite the required use of HPDs, NIHL continues to be a problem for workers in a variety of industries, as well as for military personnel. Compliance (including correct HPD fitting and use) as well as unexpected unprotected exposures (in cases where noise was unexpected or individuals chose not to use HPDs) remain significant obstacles to hearing protection. Moreover, there are some unique and excessive noise conditions for which HPDs are inadequate even when used correctly and consistently. Although HPDs can provide robust protection, there has been significant interest in alternative therapeutic-based strategies for protection given the aforementioned challenges with appropriate use and situations where HPDs are insufficient. These challenges perhaps provide some insight into why NIHL remains one of the most common occupational diseases and the second most self-reported occupational injury or illness (NIOSH 2001). Industries with high numbers of noise-exposed workers include agriculture, mining, construction, manufacturing and utilities, transportation, and the military.

The problems and challenges of NIHL are global. According to one report, 16 % of disabling hearing loss in adults worldwide is attributed to occupational noise, with 7–21 % affected across different geographical regions (Nelson et al. 2005). Harmful recreational exposure from music listening, hunting, and other nonwork-related exposure add significantly to this incidence. There are a wide variety of affected individuals. There are significant social, emotional, economic, and quality of life costs of NIHL for not only those individuals, but also, government agencies and health care providers. Given these costs, identifying novel therapeutic agents that effectively attenuate NIHL would be a significant healthcare advance, with the potential to add new hearing conservation "tools" to the hearing conservation toolkit.

Improved understanding of the harmful effects of metabolic stress on outer hair cells and identification of agents that reduce metabolic stress and preserve hearing in animal models have increased enthusiasm for these approaches, and a small number of individual, randomized, placebo-controlled, double-masked studies have been completed. However, the FDA has not approved any agent for use in the prevention of NIHL. New data from additional studies are required in order to provide the evidence base needed to drive changes in our approach to hearing conservation, with new therapeutics hopefully becoming an indispensable tool with which we can approach the task of protecting worker hearing, particularly in cases where HPDs just do not provide adequate protection.

If (or, perhaps, when) any new prescription medication options become available, workers interested in these treatments will need to be referred to a physician for consultation and possible prescription. Perceived boundaries may be less clear for over-the-counter (nonprescription) dietary supplements or other agents that affected individuals may seek advice on. Unfortunately, many physicians receive little formal training with respect to nutritional supplements, and thus, the role of physicians in advising supplements can be complicated. One strategy is to recommend that individuals seek a physician that specializes in hearing conservation, or more broadly, occupational health. Knowledge and understanding of the potential for health benefits is still evolving. Although supplements are available over the counter, we encourage referral to physicians for professional medical advice regarding the use of any such supplement. There are some known cases where high levels of specific supplements would be contraindicated, and any advice on medical outcomes is appropriately under the purview of a supervising physician with the appropriate medical training. Obviously robust clinical data from human trials would go a long way in alleviating many although not all of the questions. In closing, there are exciting opportunities for future changes in strategies for the prevention of NIHL, for developing better clinical models to address NIHL, and for the prevention of oxidative stress using supplements or pharmaceuticals.

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Part V
Oxidative Stress and Drug-Induced Hearing Loss

Chapter 10
Aminoglycoside-Induced Oxidative Stress: Pathways and Protection

Leonard P. Rybak and Michael J. Brenner

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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Chapter 11
Hearing Loss After Cisplatin: Oxidative Stress Pathways and Potential for Protection

Göran Laurell and Pernilla Videhult Pierre

Abbreviations

ERK	Extracellular-signal-regulated kinase
GSH	Reduced glutathione
GST	Glutathione *S*-transferase
IHC	Inner hair cell
iNOS	Inducible nitric oxide synthase
MHC	Monohydrated cisplatin complex (*cis*-diammineaquachloroplatinum(II) or *cis*-diamminechlorohydroxyplatinum(II))
NF-κB	Nuclear factor κB
NOS	Nitric oxide synthase
NOX	NADPH oxidase
OHC	Outer hair cell
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
STAT1	Signal transducer and activator of transcription-1
TNF-α	Tumor necrosis factor-α

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11.1 Overview

Cisplatin (*cis*-diamminedichloroplatinum(II) (*cis*-[$Pt(Cl_2)(NH_3)_2$])) is an old antineoplastic drug and widely used in oncology practice throughout the world. It was early established that the major antineoplastic effects of cisplatin are mediated through formation of DNA-intrastrand cross-links. Additional important non-DNA-dependent cytotoxic effects were later demonstrated. In tumor cells, resistance to cisplatin can develop, making the drug less efficacious and thereby limit its clinical use. Cisplatin causes significant toxicity, which may also limit the clinical use of the drug. One major side-effect is ototoxicity, i.e., toxic damage to the auditory end organ. Hearing loss is primarily seen in the high frequencies and is dependent on dose and individual susceptibility. At the cellular level in the cochlea, development of cisplatin-induced injury has to be viewed as a multistep process involving uptake of the drug, toxic reactions, cell injury and/or cell death, and loss of function. As the sensory cells and their supporting cells in the cochlea are terminally differentiated, non-DNA-dependent mechanisms contribute toxic effects in the inner ear via oxidative stress pathways. Although experimental studies have shown that oxidative stress is involved in cisplatin ototoxicity, these pathways are not fully understood. Moreover, the significance and molecular mechanisms of oxidative stress in the human cochlea remain to be elucidated. The discovery of oxidative stress as an important contributor to cisplatin ototoxicity has led to a number of efforts in laboratories around the world to demonstrate antioxidative otoprotection. In experimental systems, several substances have shown efficacy in preventing cisplatin ototoxicity. These findings are promising and may be of use for development of new medical interventions in order to avoid cisplatin-induced hearing loss. However, there is still a clear gap between experimental findings and otoprotection in cancer patients, as to date no single pharmacological prevention has shown convincing effects in clinical practice, and concerns remain regarding potential drug interactions that might compromise cisplatin's therapeutic efficacy.

11.2 History of Cisplatin

Medical oncology has its roots in the use of nitrogen mustard, an alkylating agent once used for treatment of hematologic malignancies. Cisplatin is regarded as an alkylate-like agent because the drug damages DNA. The discovery of the antineoplastic effects of cisplatin for treatment of solid malignancies is a major achievement in medical history and has helped thousands of patients struggling with cancer. In 1965, Rosenberg reported in Nature that electrolysis of platinum electrodes in ammonia buffer gave soluble platinum products inhibiting division of *Escherichia coli* (Rosenberg et al. 1965). This was the beginning of a new era in medical oncology which continues today. Cisplatin is the first generation of platinum-based antineoplastic drugs and has been followed by the development of carboplatin and oxaliplatin, a second and third generation platinum drug, respectively. Cisplatin is

today still a mainstay in the treatment of a number of solid cancers. The ototoxic effect of cisplatin was revealed in a phase I clinical trial and reported by Piel and Perlia (1975).

11.2.1 Review of Cisplatin Treatment

Reports on highly promising effects of cisplatin treatment for testicular cancer were presented by Highby et al. (1974). Einhorn and Donohue then demonstrated in 1977 a remarkable response in a cohort of 50 men with disseminated testicular cancer (Einhorn and Donohue 1977). Cisplatin was approved for treatment of patients with testicular cancer and ovarian cancer by the US Food and drug administration in 1978. Today, cisplatin is used for treatment of a number of different adult solid malignant tumors. Major indications include squamous cell carcinoma of the head and neck, testicular, ovarian, bladder, esophageal, nonsmall cell lung, and small cell lung cancer. Cisplatin is also regarded as an important drug component in many protocols for pediatric malignancies, such as medulloblastoma, neuroblastoma, and osteosarcoma. Cisplatin can be used alone or in combinations, with other cytostatic drugs together and with other treatment modalities, i.e., radiotherapy and surgery. In otolaryngology, cisplatin is used in combination with radiotherapy for advanced squamous cell carcinoma of the head and neck. Cisplatin is given to these patients either as induction chemotherapy or concomitantly when cisplatin and radiotherapy are given simultaneously.

In the literature dealing with the ototoxic effects of cisplatin, the drug is most often described as effective in the treatment of cancer. This is by far not always the situation. Patients struggling with cancer are normally exposed to a complex treatment where disease and cancer therapy affect a number of physical and psychological functions. Moreover, the antineoplastic effects induced by cisplatin-based chemotherapy are not always dramatic in terms of cure or survival. One important limitation is drug resistance; after initial treatment, tumors may develop resistance to cisplatin which may lead to uncontrolled tumor growth and thereby tumor cell resistance becomes a major obstacle for further successful treatment.

Although the therapeutic arsenal in medical oncology has taken new steps with the use of molecular target therapy, cisplatin still has a central role in oncologic treatment. Preclinical and clinical studies are now also being undertaken to identify if cisplatin can be combined with these new classes of drugs in order to improve the therapeutic efficacy with acceptable side-effects.

11.2.2 Mode of Cisplatin Administration

Cisplatin in most patients is administered intravenously. However, peritoneal carcinomatosis is an indication for intraperitoneal administration. Other cisplatin delivery systems may be under development. One important aspect to take into

consideration when studying tumor reduction and toxic side-effects of cisplatin is the variety of treatment schedules with differences in dose, interval between drug administrations, and number of courses. The mode of drug administration affects the pharmacokinetics of the drug and most probably the uptake of the drug in the inner ear (Ekborn et al. 2000). Very little is known about how the pharmacokinetics influence the ototoxic side-effects of cisplatin. One limitation with many pharmacokinetic studies is the nonselective technique used for quantification of the drug. Using a selective technique for analysis of cisplatin, Ekborn et al. demonstrated in a guinea pig model that the peak concentration was of less importance than the area under the concentration-time curve for change in hearing thresholds measured using electrophysiological techniques (Ekborn et al. 2000). There is reason to speculate that the mode of administration and period of time between cisplatin courses modulate mechanisms of toxic action in the inner ear, e.g., on the production of free radicals and thereby effects on the redox systems.

11.2.3 Antineoplastic Effects

Although cisplatin has been used for four decades in clinical settings, its cytotoxic mechanisms are not fully defined. The underlying basis for the cytotoxicity is cisplatin's reactivity and ability to bind to many different molecular sites. Two major mechanisms have been proposed, DNA-dependent mechanisms, causing inhibition of transcription, and non-DNA-dependent mechanisms, involving mitochondrial damage.

11.2.4 Adverse Effects

An adverse effect is an unwanted and harmful reaction. It is an obligation for the medical care providers and institutions to identify adverse effects and estimate their incidence and severity in order to achieve best medical practice. Chemotherapy is more or less always associated with adverse effects affecting vital functions and quality of life. The severity of adverse reactions induced by chemotherapy ranges from life threatening to mild. The management of adverse reactions in cancer patients is an important part of oncology practice. Besides the medical problem that adverse reactions create, they may also introduce ethical dilemmas, especially in palliative care. Cisplatin is not an exception; the drug causes a wide range of adverse effects. These include nephrotoxicity, gastrointestinal reactions (nausea and vomiting), ototoxicity, myelosuppression, and vascular insults. Nephrotoxicity is regarded as the major adverse effect and can be life-threatening and renal function is therefore always controlled before the start of cisplatin treatment. As cisplatin has been used for such a long time, there is an existing data base of the incidence and severity of these adverse effects and also how to mitigate the most important and frequent adverse effects; however, this is not the case for ototoxic side-effects.

Cisplatin nephrotoxicity can be prevented or reduced (but not in all patients) by vigorous hydration and mannitol-induced diuresis. To date there is no preventive measure for ototoxic side-effects effective in clinical practice.

11.2.5 Cisplatin-Induced Hearing Loss

Damage to the human inner ear produced by cisplatin can be manifested as tinnitus and/or hearing loss, primarily at high frequencies (Laurell and Borg 1988; Laurell and Jungnelius 1990; Schaefer et al. 1985). There are very few reports of vestibular symptoms in association with cisplatin treatment. A number of experimental and clinical studies have shown that cisplatin has a rather rough dose-dependence for ototoxic effects. High cumulative doses (Laurell and Jungnelius 1990) and high single doses (Laurell and Jungnelius 1990; Schaefer et al. 1985) are reported to produce more severe ototoxic symptoms. As there is a variety of dose schedules used in oncology practice, it is recommended that hearing effects are identified for each treatment schedule. While cisplatin ototoxicity is a clinical problem, it has to be pointed out that this is not the case for every patient with a toxic cochlear injury. Studies screening cisplatin-induced hearing loss related to quality of life are needed to provide a realistic understanding of the clinical importance of this problem. As cancer patients treated with cisplatin usually have a complex symptom burden, general hearing quality of life data generated from non-malignant patient cohorts cannot be applied to patients affected by cisplatin-induced hearing loss. Hearing disability induced by cisplatin in pediatric cancer patients is of special interest as these patients now have a high rate of survival and long-expected duration of survival with a hearing handicap. One important aspect of prevention of cisplatin-induced hearing loss is the possibility of predicting susceptibility before cisplatin-based chemotherapy. No standard methodology exists, which provides a medical dilemma in the handling of patients receiving cisplatin. Monitoring hearing function with pure-tone audiometry before and between treatment courses is one generally accepted tool that can be used for identification of hearing loss. However, a sudden and severe hearing loss may occur which cannot be properly identified by repeated audiometry. High-frequency screening or DPOAE might be useful in early detection of cisplatin-induced hearing loss in selected cases. However, for the majority of patients with cancer undergoing cisplatin treatment, hearing thresholds above 8 kHz are difficult to assess and are also of limited value for the planning of further therapy.

11.2.6 Effects in the Inner Ear

It is of fundamental importance to fully understand the mechanisms of cisplatin's toxic action in the inner ear to counteract its ototoxicity. This knowledge of the effects of cisplatin on the human inner ear is lacking. As cisplatin is a very reactive substance, generalization of preclinical results to the human cochlea must be done

with care; e.g., findings from experimental animals and organ of Corti cultures may be greatly influenced by extreme concentrations of cisplatin, far above those occurring in the human inner ear. There are also a number of confounding factors in cancer patients that may affect the hearing results obtained in human studies, such as nutritional status (Blakley et al. 1994) and radiotherapy to the skull base (Miettinen et al. 1997). With this in mind, the present overview of cisplatin's effects in the inner ear will basically include discoveries from the laboratory dating from the 1970s when first efforts were made to understand the complex action of cisplatin on the inner ear until today.

11.3 Morphological Changes

Outer hair cell (OHC) loss is most often regarded as the major and characteristic morphological change induced by cisplatin and is frequently used as a primary outcome measure when studying otoprotection. Nevertheless, cisplatin acts on several additional cochlear sites and other cisplatin-induced morphological changes at the cellular level can be observed by light microscopy as well as scanning electron microscopy (Comis et al. 1986; Laurell and Bagger-Sjöbäck 1991a, b). The first thorough evaluation of cisplatin effects on the hearing end organ in primates was presented by Stadnicki et al. (1975). Following daily cisplatin injections, OHC loss in the lower (basal) cochlear turns was found (Stadnicki et al. 1975). Similar results were later reported in the human cochlea (Wright and Schaefer 1982; Strauss et al. 1983; Engström et al. 1987). The susceptibility of OHCs has been demonstrated in a number of experimental studies. A general finding is that the OHCs in the first row of the basal turn are most vulnerable (Laurell and Bagger-Sjöbäck 1991a, b; Böheim and Bichler 1985; Berglin et al. 2011). Injury to inner hair cells (IHCs) is rarely reported at reasonable dose levels. However, IHC loss has been observed in the golden hamster (Melamed et al. 2000). There is some experimental evidence that morphological injury to the supporting cells precede injury to the OHCs (Laurell and Bagger-Sjöbäck 1991a, b; Estrem et al. 1981; Ramirez-Camacho et al. 2004). Evidence for involvement of the strial vascular architecture in the toxic processes that is associated with cisplatin injury also has been found in some human and experimental studies (Tange 1987; Sluyter et al. 2003; Komune et al. 1981; Laurell et al. 2007). A temporary or permanent lesion to the stria vascularis corresponded to the decline of the endocochlear potential observed in some electrophysiological studies on cisplatin ototoxicity (Sluyter et al. 2003; Komune et al. 1981; Laurell and Engström 1989a, b). In experimental animals surviving for 28 days, this decline of the endocochlear potential was found to be reversible (Hamers et al. 2003). Loop-inhibiting diuretics such as furosemide at high doses strongly potentiate the effect of cisplatin on the endocochlear potential (Li et al. 2011; Laurell and Engström 1989a, b). Apart from its toxic effects on the organ of Corti and stria vascularis, there are also data showing that spiral ganglion cells may be a unique target for cisplatin (Li et al. 2006). At very high doses, a number of in vivo studies have

shown a total disruption of the organ of Corti morphology (Laurell and Bagger-Sjöbäck 1991a, b; Komune et al. 1981; Nader et al. 2010).

Taken together, a number of specific morphologic changes in the cochlea have been observed in vivo after cisplatin treatment. If cisplatin interacts with all these targets as a primary toxic event or if there is a cascade of events starting with a toxic process to a specific cell type remains unknown. Because of the complex tissue structure in the cochlea, it is difficult to reveal the sequences of acute injury by morphological evaluation. The pattern of cell injury needs to be better defined in order to fully understand cisplatin ototoxicity.

11.3.1 Inner Ear Pharmacokinetics

Pharmacokinetic characteristics are important to consider in order to understand cisplatin-induced ototoxicity. As cisplatin readily undergoes biotransformation and reacts covalently with plasma protein binding sites in the blood compartment, the level of free drug quickly decreases after intravenous administration; the elimination half-life of cisplatin in the systemic circulation is approximately 30 min (Andersson et al. 1996; Reece et al. 1989; Cashin et al. 2013; Hellberg et al. 2009). However, elevated platinum levels can be measured in patients years after termination of cisplatin chemotherapy (Gietema et al. 2000). Cisplatin is easily subjected to ligand-exchange reactions with nucleophiles, resulting in platinum complexes with e.g., albumin (Gullo et al. 1980), globulin (Gullo et al. 1980), cysteine (Andrews et al. 1984), glutathione (GSH) (Andrews et al. 1984), and methionine (Andrews et al. 1984). These biotransformation products are generally less active than the parent compound (Andrews et al. 1984; Daley-Yates and McBrien 1984; Dedon and Borch 1987; Muldoon et al. 2001; Shirazi et al. 1996). Cisplatin is also spontaneously hydrolyzed (Miller et al. 1991; Miller and House 1989a, b, 1990, 1991; Ehrsson et al. 1995). In contrast to other types of biotransformations, hydrolysis constitutes an activation pathway of cisplatin (Shirazi et al. 1996; Daley-Yates and McBrien 1984; Litterst 1981; Ekborn et al. 2003a, b; Yachnin et al. 1998). The hydrolysis is suppressed by a high chloride concentration, and increasing the sodium chloride concentration of the cisplatin vehicle decreases the toxicity in cisplatin-treated animals (Litterst 1981; Daley-Yates and McBrien 1985), indicating the significance of hydrolysis of cisplatin in vivo. In vitro, a number of different hydrolysis products can form, such as the dihydrated cisplatin complex (Miller and House 1990; Bancroft et al. 1990; Segal and Le Pecq 1985), which is extremely toxic when protonated, as shown in experimental animals (Cleare and Hoeschele 1973). However, in vivo, monohydrated cisplatin complex (*cis*-diammineaquachloroplatinum(II) or *cis*-diamminechlorohydroxyplatinum(II)) (MHC) (Fig. 11.1) is considered the prevailing hydrated species, (Miller and House 1990; Bancroft et al. 1990; Segal and Le Pecq 1985). MHC has a p*K*a of about 6.6 (Andersson et al. 1994), which means that even a slight lowering of the pH from normal physiological levels will result in a

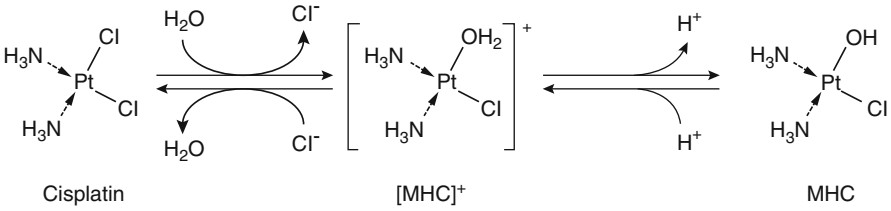

Fig. 11.1 In vivo, cisplatin (*left*) is hydrolyzed to the monohydrated cisplatin complex (MHC; *middle* and *right*), which is highly reactive when protonated ([MHC]$^+$; *middle*)

large increase of the fraction of [MHC]$^+$ (Fig. 11.1), a product that is much more reactive than uncharged MHC (Yachnin et al. 1998; Andersson et al. 1994). This would seem to explain why the cytotoxicity of cisplatin increases with decrease in pH (Tanaka et al. 2004; Murakami et al. 2001; Atema et al. 1993).

When performing kinetic studies of cisplatin, it is of course important to be able to separate active from inactive platinum compounds, which is not an easy task. Another obstacle is that there is no standard methodology for the study of pharmacokinetics in the human inner ear. A third consideration is that only tiny volumes can be sampled from the inner ear due to the small dimension of the inner ear compartments and the risk of contamination of the samples with cerebrospinal fluid (Hara et al. 1989; Salt et al. 2006). Ehrsson and coworkers performed pharmacokinetic studies using a liquid chromatographic technique for the selective analysis of intact cisplatin in perilymph samples from experimental animals treated with cisplatin (Hellberg et al. 2009, 2013). They found that in samples of scala tympani aspirated from the basal turn in guinea pigs, the concentration of cisplatin peaked within half an hour after a 3-min intravenous infusion of cisplatin (Hellberg et al. 2009, 2013). With a cisplatin dose of 8 mg/kg body weight, the maximum concentration of cisplatin in scala tympani from the basal turn was approximately 6 μM (Hellberg et al. 2013). Compared to blood, cisplatin showed a delayed elimination from scala tympani perilymph within the first hours after administration (Hellberg et al. 2009, 2013), after which the concentration of cisplatin was below the level of detection (Hellberg et al. 2013).

In contrast to cisplatin, the third generation platinum drug oxaliplatin is not ototoxic in vivo. Unfortunately, oxaliplatin cannot substitute cisplatin in the clinic in order to avoid ototoxicity since its antitumoral spectrum differs from that of cisplatin. The diverging ototoxic profiles are likely explained by the different pharmacokinetics of the drugs; the area under the concentration-time curve of intact drug in scala tympani perilymph after oxaliplatin administration was half of that obtained after equimolar cisplatin administration (Hellberg et al. 2009). An even larger difference was found in total cochlear platinum content (Hellberg et al. 2009). It is still not clear how cisplatin is transported to the inner ear. Recent data (reviewed by Waissbluth and Daniel 2013) suggests the involvement of copper transport 1 (More et al. 2010) and the organic cation transporter 2 (More et al. 2010; Ciarimboli et al. 2010).

11.3.2 Oxidative Stress Pathways in Cell Lines and Cisplatin Ototoxicity

Most of our profound knowledge on oxidative stress induced by cisplatin comes from findings in different tumor cell lines. Several in vitro studies have also shown an increased production of reactive oxygen species (ROS) in rodent auditory neurons (Gabaizadeh et al. 1997), cochlear explants (Clerici et al. 1996), and auditory cell lines (Chang et al. 2013) following direct exposure to cisplatin. In general, very high concentrations of cisplatin have been used to study these redox effects. Even though in vitro studies offer well-characterized models for cisplatin-induced cell injury, their use can be limited by the fact that the vascular system and intracochlear transport systems of the drug is circumvented. For example, Laurell and coworkers showed that an equimolar dose of cisplatin and its analog oxaliplatin induced equal toxicity in OHC cultures, whereas in vivo, systemic cisplatin administration was ototoxic while an equimolar systemic dose of oxaliplatin was not (Hellberg et al. 2009). Another factor to consider when assessing the effects of cisplatin in vitro is the biotransformation of the drug, which may differ from the in vivo situation. To understand the complexity of oxidative stress pathways induced by cisplatin in the mammalian cochlea, different approaches are needed and findings that merge in vivo and in vitro studies have the potential to increase knowledge on the pathophysiology.

In vitro studies have suggested that cisplatin can interfere with the ubiquitous glutaredoxin system (Gabaizadeh et al. 1997; Sha et al. 2001; Kopke et al. 1997). Van De Water and colleagues showed in organ of Corti explants that cisplatin-induced cytotoxicity was associated with a reduction of GSH levels and an accumulation of hydrogen peroxide (H_2O_2) and that treatment with the GSH inhibitor L-buthionine sulfoximine enhanced the cytotoxic effects (Kopke et al. 1997). Cisplatin has a well-known propensity to react with thiols such as GSH (Gullo et al. 1980; Andrews et al. 1984), which may in part explain the reduced GSH levels induced by cisplatin.

So and coworkers showed that cisplatin caused cytotoxicity in HEI-OC1 auditory cells (Kim et al. 2010; So et al. 2007) and rat primary organ of Corti explants (Kim et al. 2010) through the generation of ROS, a likely source being NADPH oxidase 1 (NOX1) and/or NADPH oxidase 4 (NOX4) (Kim et al. 2010). NOX1 and NOX4 are members of the NOX family, which is composed of transmembrane isoenzymes that reduce oxygen to the free radical superoxide ($O_2^{\bullet -}$) (Bedard and Krause 2007). Once produced, superoxide can generate other ROS or reactive nitrogen species (RNS), such as hydrogen peroxide (H_2O_2) by dismutation and peroxynitrite ($ONOO^-$) by reaction with nitric oxide (Bedard and Krause 2007). Hydrogen peroxide can, in turn, readily generate the potent hydroxyl radical ($^{\bullet}OH$) in the presence of transition metals, such as iron (Bedard and Krause 2007). Increased hydroxyl radical formation induced by cisplatin has been demonstrated by Clerici et al. in a study on cochlear explants (Clerici et al. 1996). So and colleagues also showed that the induction of NOX1 and NOX4 was due to upstream activation of proinflammatory cytokines, especially tumor necrosis factor-α (TNF-α), the activation of which

was dependent on the extracellular-signal-regulated kinase (ERK) and nuclear factor κB (NF-κB) (Kim et al. 2010; So et al. 2007). Krause and coworkers have suggested that another member of the NOX family, NOX3, is important for cisplatin-induced ototoxicity; they found NOX3 to be highly expressed in the organ of Corti and spiral ganglia of rodents and that cisplatin enhanced the NOX3-dependent generation of superoxide in vitro (Banfi et al. 2004). In accordance with these findings, Rybak and colleagues discovered that cisplatin increased the expression of NOX3 and ROS in the organ of Corti hair cell line UB/OC-1 (Mukherjea et al. 2008). They subsequently showed that this cisplatin-induced ROS generation involved activation of the signal transducer and activator of transcription-1 (STAT1) (Kaur et al. 2011), a cytoplasmic transcription factor involved in signaling cascades initiated by cytokines and cellular stress. Besides STAT1, induced expression of the inflammatory mediators inducible nitric oxide synthase (iNOS), cyclooxygenase-2, and TNF-α as well as of the immune cell markers CD14 and CD45 was found (Kaur et al. 2011).

Besides the glutaredoxin system, the thioredoxin defense system plays an important role against oxidative stress. The importance of thioredoxin reductase as a potential molecular target in cisplatin ototoxicity has recently been shown in the organ of Corti cell culture (Dammeyer et al. 2014). The predominantly cytosolic isoenzyme thioredoxin reductase 1 has a reactive nucleophilic selenocysteine residue which is readily derivatized by cisplatin (Arner et al. 2001). This may be a desirable therapeutic effect in the case of cancer therapy but it is unwanted in the inner ear and may contribute to the ototoxic effect of cisplatin.

11.3.3 Oxidative Stress Pathways in Experimental Animals and Cisplatin Ototoxicity

The homeostasis of the cochlea is dependent upon antioxidant system activity. Overproduction of ROS alters redox balance and signaling function, which may activate apoptosis mechanisms, eventually causing cell death and loss of function. Several experimental animal studies have found increased cochlear levels of the oxidative stress markers malondialdehyde (Whitworth et al. 2004; Teranishi et al. 2001; Ravi et al. 1995; Campbell et al. 2003a, b; Qu et al. 2012; Xiong et al. 2011; Rybak et al. 1995, 1999, 2000) and 8-iso-prostaglandin F2α (8-iso-PGF2α) (Qu et al. 2012) in rodents treated with cisplatin. But what are the underlying mechanisms?

Several in vivo studies suggest that cisplatin causes depletion of the glutaredoxin system. In studies on rodents, cisplatin reduced the cochlear levels of GSH (Ravi et al. 1995; Rybak et al. 1999, 2000; Lautermann et al. 1997) glutathione peroxidase (Ravi et al. 1995; Rybak et al. 1999, 2000), glutathione reductase (Ravi et al. 1995; Campbell et al. 2003a, b; Rybak et al. 1999, 2000), and glutathione *S*-transferase (GST) (Lautermann et al. 1997). Rybak and coworkers have suggested that cisplatin caused an overall decrease in GSH levels rather than an increased GSH oxidation rate, since they were unable to detect oxidized glutathione (i.e., glutathione disulfide)

(Ravi et al. 1995), in agreement with a later study performed by the same research group (Rybak et al. 2000). The decrease in GSH could be due to the proclivity of cisplatin to react with GSH, as discussed in a previous section. Rybak and coworkers have also found reduced cochlear levels of superoxide dismutase and catalase in cisplatin-treated rats (Campbell et al. 2003a, b; Rybak et al. 1999, 2000), whereas González-García et al. instead found increased cochlear levels of superoxide dismutase in the same species (Gonzalez-Garcia et al. 2010). In the former studies, a much higher dose of cisplatin was administered than in the latter study, 16 versus 5 mg/kg. Possibly, when given in a very high dose, cisplatin exhausts the redox systems whereas when given in a more moderate dose, triggering antioxidant effects can instead be detected. However, in one study by Rybak and coworkers, increased levels of superoxide dismutase as well as catalase were found, for unknown reasons (Ravi et al. 1995).

As previously mentioned, the mammalian inner ear has been shown to express very high levels of NOX3 (Banfi et al. 2004). Rybak and colleagues demonstrated that large doses of cisplatin in rats caused elevated cochlear expression of NOX3 (Mukherjea et al. 2006, 2008, 2010). The increase could be localized to OHCs (Mukherjea et al. 2008, 2010), spiral ganglion cells (Mukherjea et al. 2008, 2010), stria vascularis (Mukherjea et al. 2008, 2010), and supporting cells (Mukherjea et al. 2008), thus structures that are known to be susceptible to damage by cisplatin. So and coworkers demonstrated elevated cochlear expression of NOX3 as well as of NOX1 and NOX4 in cisplatin-treated mice (Kim et al. 2010). The high levels of NOX1 and NOX4 were localized to the spiral ligament, spiral limbus, spiral ganglion neurons, OHCs, and IHCs, whereas low levels were found in the stria vascularis (Kim et al. 2010). It is noteworthy that the cellular site of ROS production is not established. Even though the OHCs are mostly damaged by cisplatin, ROS may be produced by different cochlear cell types as this is a general phenomenon of many mammalian cells.

Rybak and coworkers found that NOX3-generated ROS in cochleae from cisplatin-treated rats caused activation of STAT1 (Kaur et al. 2011). Cisplatin-induced STAT1 activation was found in OHCs, stria vascularis, and spiral ganglion cells (Kaur et al. 2011). Besides STAT1, cisplatin-induced expression of iNOS, cyclooxygenase-2, TNF-α, CD14, and CD45 was found (Kaur et al. 2011). In agreement with those results, So et al. found that cisplatin treatment caused elevation of TNF-α throughout the stria vascularis, spiral ligament, spiral limbus, modiolar spiral veins, and lacunae, and the organ of Corti in rats (So et al. 2007). The expression of the proinflammatory cytokines IL-1β, and IL-6 were also elevated but less ubiquitously (So et al. 2007). The elevated expressions likely involved activation of the transcription factor NF-κB (So et al. 2007).

iNOS and other NOS isoenzymes generate the free radical nitric oxide (•NO) by metabolizing L-arginine to L-citrulline. Nitric oxide readily reacts with superoxide, producing the reactive peroxynitrite ($ONOO^-$), which, in turn, can generate other, even more potent ROS/RNS, such as nitrosoperoxycarbonate ($ONOOCO_2^-$) and the hydroxyl radical. Elevated nitric oxide in cochlear tissue following cisplatin treatment has been found in rodents (Xiong et al. 2011; Kelly et al. 2003). Using higher

doses of cisplatin, Watanabe et al. (2000a, b) and Kaur et al. (2011) increased the cochlear expression of iNOS in rodents (Kaur et al. 2011; Watanabe et al. 2000a, b). The expression was mainly localized to the stria vascularis, spiral ganglion cells, and the organ of Corti, but not to the IHCs and OHCs (Watanabe et al. 2000a, b). It has also been shown that cisplatin treatment of mice induced the expression of iNOS in the lateral wall, an alteration which was accompanied by increased levels of NF-κB and, in stria vascularis, ssDNA (Watanabe et al. 2002).

In conclusion, findings from experimental animals show that cisplatin treatment can cause oxidative stress in the cochlea, e.g., due to induction of NOX, depletion of the glutaredoxin system, and/or induction of iNOS. The oxidative stress may be a consequence of toxic platinum-DNA adducts formed by cisplatin and/or MHC. However, oxidative stress can likely be generated independently of platinum-DNA adducts due to the avidity of cisplatin and/or MHC to react with nucleophiles involved in the cellular antioxidant defense, e.g., GSH.

11.4 Evidence for Importance of Oxidative Stress in Cisplatin Ototoxicity in the Human Cochlea

There are no known methods to study specific biochemical toxic processes in the cochlea of patients during cisplatin treatment; our tools are limited to functional monitoring. Moreover, very few studies based on examinations of the human inner ear postmortem have been published. Thus, there is a lack of clear-cut evidence that cisplatin-induced hearing loss in patients is associated with oxidative stress. Thus, the primary evidence for the key role of oxidative stress in cisplatin-induced ototoxicity comes from animal laboratory studies.

There are some clinical data implying that the ototoxic effects of cisplatin might depend on interindividual variabilities in redox systems. The GST supergene family encodes isoenzymes that catalyze the detoxifying reactions of GSH. Some GST loci are polymorphic, demonstrating alleles that are null, encode low-activity variants, or are associated with variable inducibility. In a Norwegian study on testicular cancer long-term survivors that had received cisplatin-based therapy, Oldenburg et al. discovered that the genotype 105Val/105Val-GSTP1 was associated with better hearing than 105Ile/105Ile-GSTP1 and 105Val/105Ile-GSTP1, especially when combined with GSTT1*1 (wild genotype) and GSTM1*1 (Oldenburg et al. 2007). In a study on cisplatin-treated Asian pediatric cancer patients, Choeyprasert et al. found that subjects with GSTT1*1 had a significantly increased risk of hearing impairment compared to those with GSTT1*0 (null genotype), whereas no significant association was found with GSTM1 polymorphism (Choeyprasert et al. 2012). Peters et al. found that the GSTM3*B allele was more common among subjects with normal hearing after cisplatin therapy than among those with deteriorated hearing (Peters et al. 2000), while Ross et al. did not find any associations at all between GST polymorphism and hearing loss (Ross et al. 2009). It is possible that

otoprotection by a certain genetic pattern is due to physiological functions not related to the inner ear, such as a higher clearance of cisplatin and/or MHC in the kidneys, leading to less cisplatin/MHC reaching the inner ear. Clearly, more clinical research is needed within this field.

11.4.1 Methods for Otoprotection/Potential for Protection

Methods for otoprotection of cisplatin treatment have represented a key goal for more than two decades. There are certainly a number of methods identified from animal studies that can be transferred to clinical trials. However, in the search for otoprotective measures, we must be sensitive and consider associated medical and ethical issues. These problems must be identified and well-evaluated before a clinical trial is undertaken. One major consideration is that cisplatin-based chemotherapy is given to individuals with malignant disease and therefore no otoprotective intervention ought to interfere with the cancer treatment. Another consideration is that there should be no risk for harm to the inner ear produced by the otoprotective intervention. Moreover, the risk for ototoxic side-effects and the indication for cisplatin-based chemotherapy have to be identified in each patient receiving the drug, with consideration of the curative or palliative intention. Otoprotection should be considered for high-risk individuals, in most cases patients receiving high-dose treatment. Many experimental studies on otoprotection are inadequately designed and need to be viewed critically. Others may need to be revalidated and modified before the results will be considered for a potential clinical application. High-quality clinical studies are yet needed to determine if scavengers/antioxidants can rescue or protect the hearing during cisplatin treatment. It may even be difficult in controlled randomized studies to show a significant otoprotective effect unless the otoprotection is complete.

At the moment there is no evidence from clinical studies that an otoprotective measure could be undertaken with significant protective effect and without the risk of reducing antineoplastic effects.

11.4.2 Dose Reduction/Treatment Interruption

In patients receiving high-dose cisplatin, serial audiometric evaluations are recommended. Once a significant cisplatin-induced hearing loss is established, further ototoxic side-effects may be prevented by reduction of the cisplatin dose or by changing from cisplatin to a less ototoxic drug. A cardinal disadvantage with dose reduction/treatment interruption is that it may have a negative impact on the development of the cancer disease.

11.4.3 Otoprotection by Administration of Antioxidants

Solid clinical evidence of the importance of oxidative stress for cisplatin-induced ototoxicity is still lacking due to the difficulties to investigate the human inner ear. In contrast, there are extensive preclinical data showing that cisplatin can affect the cellular redox status and thereby cause ototoxic oxidative stress. There are numerous experimental studies demonstrating that cisplatin-induced ototoxicity can be reduced by administration of an exogenous antioxidant in conjunction with cisplatin treatment, whereas similar otoprotective studies in humans are scarce. The otoprotection is usually considered to involve one or both of the following mechanisms:

1. Strengthening of the endogenous antioxidant defense.
2. Reduced cochlear levels of cisplatin and/or MHC.

The reason for the latter mechanism is that many exogenous antioxidants are prone to react with cisplatin and/or MHC (Ekborn et al. 2002, 2004; Brouwers et al. 2008), resulting in inactive platinum–antioxidant complexes (Andrews et al. 1984; Daley-Yates and McBrien 1984; Dedon and Borch 1987; Muldoon et al. 2001; Shirazi et al. 1996).

In vivo, two main methods of administrating antioxidants have been used:

1. Systemic treatment
2. Local treatment

Systemic antioxidant treatment is commonly performed by intravenous injection/infusion in humans and by intraperitoneal or, less frequently, intravenous injection/infusion in experimental animals. A major drawback with systemic treatment is that it may decrease the antineoplastic effects of cisplatin (Inoue et al. 1991), suggesting that the method is inappropriate for clinical use. Some results indicate that this problem can be solved by separation of cisplatin and the otoprotector by the time of administration (Harned et al. 2008); however, data are conflicting (Inoue et al. 1991). Another suggested solution in localized disease without metastatic spread is to use separate systemic routes of administration by giving the otoprotector intravenously and cisplatin intra-arterially (Zuur et al. 2007; Rasch et al. 2010) or intraperitoneally (van Rijswijk et al. 1997).

Another way to reduce the risk of decreased anticancer effects is to employ local antioxidant treatment. In experimental animals, it is possible to perform intracochlear administration (Ekborn et al. 2003b; Wang et al. 2003; Cappaert et al. 2005), a method that is unacceptable for clinical use due to the risk of iatrogenic hearing damage. In humans, inner ear drug delivery may be performed by middle ear administration, the most common technique being a simple transtympanic drug injection (Riga et al. 2013; Yoo et al. 2013; Wang et al. 2012; Berglin et al. 2011).

11.4.4 Antioxidants Used in Experimental Animals

Some of the systemically administered antioxidants that have shown otoprotective effects in experimental animals are acetylcysteine (Lorito et al. 2011; Dickey et al. 2004), amifostine (Hussain et al. 2003; Church et al. 2004), astragalosides (Xiong et al. 2011), diethyldithiocarbamate (Rybak et al. 1995), ebselen (Rybak et al. 2000; Lynch et al. 2005), glutathione ester (Campbell et al. 2003a, b), hydrogen gas (Qu et al. 2012), lipoic acid (Rybak et al. 1999; Mukherjea et al. 2008), methionine (Campbell et al. 1996, 1999, 2003a, b; Lorito et al. 2011; Reser et al. 1999; Cheng et al. 2005; Li et al. 2001, 2006; Reser et al. 1999), methylthio benzoic acid (Rybak et al. 2000; Kamimura et al. 1999), sodium thiosulfate (Dickey et al. 2005; Kaltenbach et al. 1997; Saito et al. 1997; Church et al. 1995), and α-tocopherol (Teranishi et al. 2001). Local administration of an otoprotective antioxidant is much less frequently employed, but has been successfully performed with acetylcysteine (Saliba et al. 2010; Choe et al. 2004), epicatechin (Lee et al. 2010), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (a water-soluble analog of tocopherol) (Teranishi and Nakashima 2003), methionine (Korver et al. 2002; Li et al. 2001), sodium thiosulfate (Berglin et al. 2011; Cappaert et al. 2005; Wang et al. 2003; Stocks et al. 2004), and vitamin C (Celebi et al. 2013). However, there are several reports on acetylcysteine causing inflammatory reactions when applied locally (Nader et al. 2010; Saliba et al. 2010; Choe et al. 2004).

Otoprotective effects in experimental animals have also been achieved with more selective antioxidants. NOS was targeted by intraperitoneal administration of the NOS inhibitors aminoguanidine (Kelly et al. 2003) and *N*-nitro-L-arginine methyl ester (L-NAME) (Watanabe et al. 2000a, b), respectively. Targeting of NOX pathways have been obtained with several methods: systemic (Kim et al. 2010; So et al. 2007) and transtympanic (Kaur et al. 2011) administration of the TNF-α inhibitor etanercept and transtympanic administration of NOX3 siRNA (Mukherjea et al. 2010; Kaur et al. 2011) and STAT1 siRNA (Kaur et al. 2011).

Molecular effects found in cochleae from cisplatin-treated experimental animals subjected to antioxidant-induced otoprotection are decreased ROS (Kaur et al. 2011), iNOS (Li et al. 2006), HMG1 (Li et al. 2006), malondialdehyde (Teranishi et al. 2001; Qu et al. 2012; Xiong et al. 2011; Rybak et al. 1995, 1999, 2000; Kelly et al. 2003), nitric oxide (Xiong et al. 2011), NOX1 (Kim et al. 2010), NOX3 (Mukherjea et al. 2008, 2010), NOX4 (Kim et al. 2010), STAT1 (Kaur et al. 2011), TNF-α (So et al. 2007), IL-1β (So et al. 2007), IL-6 (So et al. 2007), NF-κB (So et al. 2007), and 8-iso-prostaglandin F2α (Qu et al. 2012). In addition to decreased ROS, there are observed increases in catalase (Campbell et al. 2003a, b; Rybak et al. 1999, 2000), GSH (Rybak et al. 1995, 2000), glutathione peroxidase (Rybak et al. 1995, 1999, 2000), glutathione reductase (Campbell et al. 2003a, b; Rybak et al. 1999, 2000), and superoxide dismutase (Campbell et al. 2003a, b; Rybak et al. 1999, 2000).

11.4.5 Antioxidants Used in Humans

In humans, the antioxidants acetylcysteine (Riga et al. 2013; Yoo et al. 2013), amifostine (Fouladi et al. 2008; Katzenstein et al. 2009; Gallegos-Castorena et al. 2007; Ekborn et al. 2004; Sastry and Kellie 2005; Marina et al. 2005; Fisher et al. 2004; Planting et al. 1999; Kemp et al. 1996), diethyldithiocarbamate (Berry et al. 1990; Gandara et al. 1995), GSH (Parnis et al. 1995), and sodium thiosulfate (Zuur et al. 2007; Rasch et al. 2010; van Rijswijk et al. 1997) have been investigated as potential candidates to reduce the ototoxic effects of cisplatin. Some support for otoprotection has been obtained with acetylcysteine (Riga et al. 2013), amifostine (Fouladi et al. 2008), and thiosulfate (Zuur et al. 2007; Rasch et al. 2010; van Rijswijk et al. 1997).

Thiosulfate is a naturally occurring ion in vivo with antioxidant properties (Iciek and Wlodek 2001). In the literature, several reports can be found on cancer patients with localized disease that have received intravenous thiosulfate in order to reduce the side-effects of cisplatin which, in turn, was given rather directly to the site of the tumor, e.g., into the peritoneum or into an artery feeding the tumor (see e.g., Zuur et al. 2007; Rasch et al. 2010; van Rijswijk et al. 1997; Howell et al. 1982; Robbins et al. 2000; Madasu et al. 1997; Homma et al. 2009). In two studies in head and neck cancer patients, treatment with intra-arterial high-dose cisplatin in combination with intravenous sodium thiosulfate resulted in similar amount of ototoxicity as intravenous high-dose cisplatin without sodium thiosulfate although the intra-arterial cisplatin dose was twice as high (Zuur et al. 2007; Rasch et al. 2010). These results are promising but more research is needed in order to verify that thiosulfate, which reacts avidly with cisplatin and MHC (Videhult et al. 2006), does not compromise the antineoplastic efficacy. Of concern are the data of Inoue et al. showing that thiosulfate administered subcutaneously as long as 72 h after systemic cisplatin treatment reduced the anticancer efficacy in nude mice (Inoue et al. 1991).

Amifostine is an organic thiophosphate prodrug that is hydrolyzed in vivo to the thiol metabolite WR-1065, which has antioxidant properties. Several investigations have been performed using systemic amifostine in order to prevent cisplatin-induced ototoxicity. One study on pediatric cancer patients found that amifostine was otoprotective (Fouladi et al. 2008), whereas most other studies did not find such an effect (Katzenstein et al. 2009; Gallegos-Castorena et al. 2007; Ekborn et al. 2004; Sastry and Kellie 2005; Marina et al. 2005; Fisher et al. 2004; Planting et al. 1999; Kemp et al. 1996). Ekborn et al. not only found that amifostine did not prevent ototoxicity, but also that it caused a decreased area under the concentration-time curve for cisplatin and MHC, indicating an unacceptable interference with the anticancer efficacy of the cisplatin treatment (Ekborn et al. 2004).

Results on local treatment with antioxidants against cisplatin-induced ototoxicity in humans are scarce (Riga et al. 2013; Yoo et al. 2013), but there are some ongoing studies, as discussed in the final section of this chapter. However, in a small study on cisplatin-treated patients, Riga et al. recently showed a slightly less worsening of hearing in ears treated with transtympanic administration of acetylcysteine, a precursor of GSH, than in control ears (Riga et al. 2013).

Table 11.1 Human studies employing local otoprotective administration found in the World Health Organization's International Clinical Trials Registry Platform with as yet no posted results

Treatment	Method	Study ID
Lactated Ringer solution	External auditory canal administration	NCT00584155
Lactated Ringer solution	Intratympanic administration via tympanostomy tube	NCT01108601
Sodium thiosulfate solution	Intratympanic administration via tympanostomy tube	NCT01369641
Methylprednisolone solution	Intratympanic injection	NCT01285674

11.5 Summary and Future Perspectives

Irreversible cochlear damage is a dose-limiting side-effect of the frequently used anticancer drug cisplatin. According to experimental animal studies, the damage involves several oxidative stress pathways, which may be targeted by administration of an antioxidant in conjunction with cisplatin therapy. A main challenge is to reduce the ototoxicity without interfering with cisplatin's antineoplastic effects. Two major routes for administration of otoprotectors are plausible, a systemic route and a local route. Systemic administration of an antioxidant seems less attractive in systemic cisplatin therapy owing to the risk for lowering of the antineoplastic effects unless the cancer disease is highly confined. Local administration of an otoprotective agent or a combination of substances offers the possibility of protecting the cochlea without compromising the antitumoral efficacy, according to preclinical data, and needs to be studied in randomized trials. Studies are ongoing investigating the clinical applicability of this approach in humans (Table 11.1).

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Chapter 12
Assessment of Interventions to Prevent Drug-Induced Hearing Loss

Jill M. Anderson and Kathleen Campbell

Abbreviations

AAA	American Academy of Audiology
ABR	Auditory brainstem response
ASHA	American Speech Language Hearing Association
ASSR	Auditory steady state responses
CIHL	Cisplatin-induced hearing loss
DIHL	Drug-induced hearing loss
DPOAE	Distortion product otoacoustic emissions
EHF	Extended high frequency
FDA	Food and Drug Administration
GLP	Good Laboratory Practices
IND	Investigational New Drug
IRB	Institutional Review Board
MDR-TB	Multi-drug resistant tuberculosis
NIHL	Noise-induced hearing loss
OAE	Otoacoustic emissions
OHC	Outer hair cells
TB	Tuberculosis
TEOAE	Transient otoacoustic emissions
VAT	Vestibular autorotation

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VEMP Vestibular evoked myogenic potential
VOR Vestibular-ocular reflex
WHO World Health Organization

12.1 Introduction to Ototoxicity

Many drugs used to treat disease are inadvertently toxic to the inner ear, i.e., they are "ototoxic." Drug-induced cellular impairments occur in the cochlea and/or vestibular structures and may subsequently manifest as hearing loss, tinnitus, disequilibrium, or a combination thereof. The classes of drugs which are most commonly associated with ototoxicity and/or vestibulotoxicity are aminoglycoside antibiotics, antineoplastic agents, loop diuretics, macrolide antibiotics, and antimalarials. The drugs most responsible for severe and irreversible ototoxic changes are the prescribed cancer fighting platinum-based antineoplastic agents and the powerful antimicrobial aminoglycosides (Rybak and Ramkumar 2007). These ototoxic drugs appear to preferentially affect either the cochlear or vestibular portions of the inner ear. Antineoplastics, predominantly the platinum compounds (cisplatin and carboplatin), are primarily cochleotoxic and aminoglycosides are not only cochleotoxic but are the most vestibulotoxic of all drugs depending on the specific aminoglycoside agent (Monsell et al. 1993). Of the aminoglycosides, kanamycin and amikacin are known to be exclusively cochleotoxic (Selimoglu 2007) while streptomycin and gentamicin are primarily vestibulotoxic (Schacht et al. 2012; Selimoglu 2007). Several over-the-counter medications may also become ototoxic at high cumulative dosing levels. Over 200 medications, both prescribed and over-the-counter, have been identified as potentially ototoxic. A partial list of some of these agents is listed in Table 12.1. Moreover, the ototoxic potential of several new drugs may not be fully known until widely used.

12.2 Drug-Induced Hearing Loss

Drug-induced hearing (DIHL) loss is typically permanent (although some cases are reversible), bilateral, and predominantly high frequency (Yorgason et al. 2006). DIHL is most often sensorineural and is typically secondary to the destruction of the cochlear outer hair cells (OHC) at the basal end of the cochlea, where high frequency sounds are encoded (Fausti et al. 1984a, b). The onset of ototoxic changes can be highly variable depending upon the pharmaceutical agent. Some ototoxic changes with cisplatin therapy occur very quickly, after only a single dose (Domenech et al. 1988), while both aminoglycoside and cisplatin therapy may cause delayed or progressive hearing loss months after drug discontinuation (Bertolini et al. 2004). Delayed ototoxicity may be, at least in part, related to the slowed clearance of some medications (i.e., antineoplastics and aminoglycosides)

Table 12.1 A partial list of ototoxic medications (adapted from the AAA (2009); Rybak (1986); Physician's Desk Reference (2013))

Antineoplastics	Aminoglycosides	Loop diuretics	Antimalarials	Macrolides	NSAIDS
Carboplatin	Amikacin	Azosemide	Chloroquine	Azithromycin	Aspirin
Cisplatin	Dihydrostreptomycin	Bumetanide	Chloroquine phosphate	Erythromycin	Etodolac
Nedaplatin	Gentamicin	Ethacrynic acid	Hydroxychloroquine		Fenoprofen
Oxaliplatin	Kanamycin	Furosemide	Quinacrine hydrochloride		Ibuprofen
Satraplatin	Netilmicin	Piretanide	Quinine sulfate		indomethacin
Vinblastine	Tobramycin	Torsemide			Naproxen
					Piroxicam
					Sulindac

from the inner ear fluids after the cessation of drug therapy (Li and Steyger 2009). The degree of hearing loss can widely vary even for the same agent and dosing (Fausti et al. 1984a, b). However, with prolonged administration or decreased clearance from cochlear fluids, OHC damage may spread from the apical high frequency area of the cochlea towards the basal low frequency end (Schacht et al. 2012), ultimately negatively impacting word recognition (Fausti et al. 1999).

The factors which contribute to the potential for ototoxic changes are the administration of concomitant medications (especially other ototoxic medications), dosage, age, hydration status, and individual genetic susceptibility to ototoxicity (Konrad-Martin and Keefe 2005). Ototoxic changes secondary to drug administration may be significantly exacerbated by exposure to noise (Li and Steyger 2009; Schacht et al. 2012). That is, the administration of ototoxic drugs may not only cause hearing loss but may also render the cochlea more susceptible to additional noise-induced hearing loss (NIHL) (Schacht et al. 2012). As for drug-induced vestibulotoxicity, oscillopsia, and disequilibrium, these conditions usually manifest from cellular damage which typically occurs in the macula of the saccule and/or crista ampullaris of the cochlear semicircular canals (Carey 2004). In fact, gentamicin is so well known for its damaging effects on the human vestibular system that it is intentionally administered transtympanically to ablate vestibular function in patients with severe unilateral dysfunction in order to ease their vertiginous symptoms (Nedzelski et al. 1993).

12.3 Ototoxic Medications

12.3.1 Aminoglycosides

Although many new antibiotics preclude the need for the administration of ototoxic aminoglycosides for many infections, aminoglycosides are still in common clinical use to treat serious Gram-negative aerobic bacterial infections when other antibiotics are ineffective or the patient is allergic to them (Yorgason et al. 2006). Although aminoglycosides are broad spectrum antibiotics (Schacht et al. 2012), they are not typically used to treat Gram-positive infections because alternate antibiotics with less toxic side effects are available for treating these infections in the United States. However, aminoglycosides are used routinely in the United States and globally for the management of acute infections common in cystic fibrosis patients (Prayle and Smyth 2010) as well as patients with multi-drug resistant tuberculosis (MDR-TB). In the United States, aminoglycosides are used as the last line of defense against some infectious diseases because they are stable against resistance when compared to other classes of antibiotics (Bassetti and Righi 2013). However, use is more widespread in developing countries.

The largest use of aminoglycosides occurs in developing countries because of their low cost (Chen et al. 2007), widespread availability, and effectiveness in the treatment of MDR-TB (Duggal and Sarkar 2007). With the World Health

Organization (WHO) reporting that one-third of the world's population is presently infected with the TB bacterium, global aminoglycoside use has become extensive and the numbers of those infected are expected to steadily increase over time (WHO 2009, 2010). Therefore, the incidence of aminoglycoside ototoxicity is also expected to increase among those treated for MDR-TB worldwide. In the absence of ototoxicity monitoring programs in most of these countries, incidence data are currently unavailable for many sites. However, Seddon et al. (2013) reviewed results from 35 studies of ototoxicity for MDR-TB worldwide and reported varying incidences ranging from 2.1 to 61.5 % depending on treatment regimen and definition of ototoxic change. In South Africa, where high-dose aminoglycosides are routinely used for MDR-TB, Harris et al. (2012) reported a 58 % incidence of ototoxic hearing loss with the majority being severe to profound. DIHL from aminoglycoside treatment is also considered to be a significant factor contributing to treatment noncompliance in these developing countries (Duggal and Sarkar 2007).

12.3.2 Cisplatin

Cisplatin (CDDP), the original platinum-based chemotherapeutic, has been effective in treating soft tissue neoplasms since first approved by the FDA (Platinol®, Bristol–Myers Squibb) for cancer treatment in the late 1970s (Blakley et al. 2002). The cancer types most often treated with cisplatin include ovarian, testicular, cervical, head and neck, lung and bladder (Rybak et al. 2007) in adults and osteosarcoma, neuroblastoma, hepatoblastoma, and germ-cell tumors in children (Langer et al. 2013). Although cisplatin typically provides powerful antitumor efficacy it also has been found to induce serious side effects such as sensorineural hearing loss, nephrotoxicity, neurotoxicity, and tinnitus (Adams et al. 1989; Blakley et al. 2002; Barabas et al. 2008). While researchers have found ways to circumvent the nephrotoxic side effects of cisplatin (Muraki et al. 2012), to date, no proven treatment has been found to prevent cisplatin-induced neurotoxicity and ototoxicity.

The incidence of cisplatin-induced hearing loss (CIHL) has been reported to highly vary in both adults and children receiving cisplatin therapy. The wide range of CIHL incidence variability for adults is largely due to differences in cumulative dosage, duration of treatment, types of assessments used to measure ototoxicity, and the scales used to rate the severity of ototoxicity. For children, an additional contributing factor to the high variability is the difficulty in assessing hearing thresholds in pediatric cancers that affect very young children (Brock et al. 1992). The median age of patients with neuroblastoma and hepatoblastoma is less than 18 months (Brock et al. 1992), an age range that can be challenging to obtain accurate hearing threshold assessments even among healthy children. The incidence of tinnitus is less well documented in adults receiving cisplatin therapy and is very difficult to assess in young children. Overall, the side effect of CIHL is of great concern in both the adult and pediatric populations undergoing cisplatin treatment. However, the

pediatric population is at greater risk for CIHL as Li et al. (2004) have reported that children younger than 5 years of age were 21 times more likely to develop significant hearing impairment when compared to older children (15–20 years).

12.4 Mechanisms of Ototoxic Medications

Because DIHL is typically irreversible, prevention is currently the best option available. Although the preservation of life is the principle goal with any drug treatment, a patient's quality of life should also be a fundamental consideration (Fausti et al. 2005; Duggal and Sarkar 2007). A better understanding of the mechanisms by which DIHL occurs at the cellular level has led to the development of several potential pharmacological agents for the protection of the inner ear from drug-induced damage. As reviewed in Chap. 10 by Rybak (aminoglycoside antibiotics) and Chap. 11 by Laurell (cisplatin), experiments in animal models have shown that after ototoxic drug administration, free radical formation occurs in the cochlea causing irreversible damage (Lee et al. 2004). This finding has led to proposed treatments with antioxidants to combat free radical formation and subsequent hearing loss in humans (see review by Campbell and Le Prell 2012; see also Chaps. 11 and 12 in this edition). Translational research investigations with prospective otoprotective pharmacological agents are currently in varying stages of preclinical and clinical development. However, none of these agents have yet completed the lengthy FDA clinical trial process and therefore, no agents are presently FDA approved to prevent and/or treat DIHL, tinnitus, and vestibular disorders as of the time of the writing of this chapter. The otoprotective agents that have been or are currently (as of January 2014) in various phases of clinic trials for DIHL (www.clinicaltrials.gov) (Table 12.2) are: D-methionine (Phase 2), Ebselen (Phase 2), *N*-Acetylcysteine (Phase 3), Sodium Thiosulfate (Phase 3), Amifostine (Phase 2).

12.5 Potential Otoprotective Agents

While some guidance exists for monitoring ototoxicity in patients (ASHA 1994; AAA 2009) no formal consensus exists for determining efficacy of a protective agent against ototoxicity in clinical trials. The procedures for assessing efficacy may vary somewhat depending on the ototoxin and the protective agent. For example, some studies may wish to address vestibular disorders or tinnitus in addition to hearing threshold shift. Further, the procedures may vary for Phase 1 clinical trials which focus on safety (Campbell et al. 2003), Phase 2 trials (therapeutic exploratory) which focus on safety and efficacy in a small group of volunteers (generally fewer than 100), and Phase 3 (therapeutic confirmatory) clinical trials which focus on safety and efficacy in large populations (100–1,000 s). Clinical trials for otoprotective agents against DIHL can be further complicated in that they must ensure that

Table 12.2 A list of interventional clinical trials for potential otoprotective agents (retrieved January 2014 with the search term "ototoxicity")

Agent	NCT number	Current clinical trial title	Phase	Sponsor	Status
Sodium thiosulfate	NCT00716976	Sodium thiosulfate in preventing hearing loss in young patients receiving cisplatin for newly diagnosed germ cell tumor, hepatoblastoma, medulloblastoma, neuroblastoma, osteosarcoma, or other malignancy	Phase 3	Children's Oncology Group	Active, not recruiting
Sodium thiosulfate	NCT01369641	The effect of sodium thiosulfate eardrops on hearing loss in patients who receive cisplatin therapy	Not known	Thomas Jefferson University	Terminated
Sodium thiosulfate	NCT00652132	Cisplatin with or without sodium thiosulfate in treating young patients with stage I, stage II, or stage III childhood liver cancer	Phase 3	Children's Cancer And Leukaemia Group	Unknown
Sodium thiosulfate	NCT00983398	Melphalan, carboplatin, and sodium thiosulfate for patients with central nervous system (CNS) embryonal or germ cell tumors	Phase 1 Phase 2	OHSU Knight Cancer Institute	Currently recruiting participants
Sodium thiosulfate	NCT00075387	Treating patients with high-grade glioma with IA carboplatin-based chemotherapy, with or without sodium thiosulfate	Phase 2	OHSU Knight Cancer Institute	Recruiting
Sodium thiosulfate	NCT00074165	Treating patients with recurrent PCNSL with carboplatin/BBBD and adding rituxan to the treatment regimen	Phase 2	OHSU Knight Cancer Institute	Terminated
Amifostine	NCT00003269	Amifostine followed by high-dose chemotherapy in treating patients with hematologic cancer or solid tumors	Phase 2	Scripps Health	Completed
N-acetylcysteine	NCT01271088	Protective effect of N-acetylcysteine against from ototoxicity	Phase 2 Phase 3	TC Erciyes University	Completed
N-acetylcysteine	NCT01131468	Prevention of drug-induced ototoxicity in peritoneal dialysis patients by N-Acetylcysteine	Phase 2	TC Erciyes University	Completed
N-acetylcysteine	NCT01138137	N-acetylcysteine given IV with cisplatin and paclitaxel in patients with ovarian cancer	Phase 1	OHSU Knight Cancer Institute	Suspended participant recruitment
Ebselen	NCT01451853	SPI-1005 for prevention and treatment of chemotherapy-induced hearing loss	Phase 2	Sound Pharmaceuticals, Incorporated	Not yet recruiting
Ebselen	NCT01444846	Otoprotection with SPI-1005 for prevention of temporary auditory threshold shift	Phase 2	Sound Pharmaceuticals, Incorporated	Ongoing, but not recruiting

the otoprotective agent does not interfere with the intended therapeutic action of the ototoxic drug under study.

Another consideration is whether the proposed otoprotective agent is for prophylaxis and, if so, if it can be given before the ototoxic drug only or if it must be continued during and/or after the therapeutic drug is administered. Some otoprotective agents are being developed as rescue agents which means they are administered after the ototoxic event but before irreversible damage has occurred. Thus, the timing of ototoxicity testing must be carefully considered not only relative to the anticipated ototoxicity of the ototoxic drug but also relative to the anticipated protective action of the otoprotective agent. Further, because some ototoxic drugs such as aminoglycoside antibiotics (Seddon et al. 2013) and cisplatin can cause progressive hearing loss after drug discontinuation (Yasui et al. 2013), some longer time points to determine if the otoprotective agent also prevents later hearing loss progression may be optimal in the clinical trials populations. Longer time points may also be needed to ensure that the otoprotective agent does not inhibit any long-term treatment benefit of the therapeutic drug such as tumor progression or recurrence as in the case of cisplatin. Given this latter long-term safety issue, the ethics of any study that does not include the possibility for long-term follow-up must be carefully considered. Long-term follow-up will be difficult, if not impossible, for many patient populations, especially in developing countries where patients may travel long distances for treatment and monitoring.

The drug delivery method for the otoprotective agent may also influence clinical trial study design and thus sample size. For example, an otoprotective agent delivered transtympanically may allow for the opposite ear of the same subject to be used as a control while that within-subject study design would not be possible for an otoprotective agent delivered systemically.

In general, clinical trials of otoprotective agents for NIHL may be progressing more quickly than for DIHL, at least in part, because for NIHL there is no risk that the otoprotective agent will reduce the therapeutic action of a target drug. Further, the clinical trial populations for prevention of NIHL are generally relatively young and healthy. This contrasts with the less healthy patient populations receiving cisplatin chemotherapy or aminoglycoside antibiotics, who also tend to be distributed preferentially among pediatric and geriatric populations in developed countries.

Interestingly, many, but not all of the otoprotective agents being developed to prevent or rescue from NIHL, show some promise for preventing DIHL so at least some of the clinical safety data from otoprotection for NIHL clinical trials may speed development of clinical trials for otoprotection from DIHL. An Investigational New Drug application (IND) to the US Food and Drug Administration (FDA) requires two species pharmacokinetic and two species toxicology studies in Good Laboratory Practices (GLP) laboratories, and may require genotoxicity studies (FDA 2007). However, once these data are collected and reviewed, they may also serve for future studies of the same otoprotective agent for other applications. Also, Phase 1a (safety evaluation in normal human volunteers) data may also translate across studies.

Eventually, we may have the opportunity to perform clinical trials for agents that reverse long standing hearing loss by regenerating cochlear hair cells, but these agents do not appear to be on the immediate horizon clinically (Collado et al. 2008; Groves 2010). The following are some pharmacologic otoprotective agents that have been in or are approaching FDA clinical trials for protection from drug-induced ototoxicity. Clinical trials allowed to move forward by the FDA are required to be posted on www.clinicaltrials.gov. That listing allows patients to volunteer to participate in clinical trials that are in progress and can provide scientists and clinicians with the opportunity to review some aspects of study design for otoprotection clinical trials. Several agents currently in clinical trials are described below.

12.5.1 D-Methionine

D-methionine, the optical isomer of L-methionine, has been found to be protective against the ototoxic effects of antineoplastics (cisplatin and carboplatin) (Campbell et al. 1996, 1999, 2007; Lockwood et al. 2000) as well as aminoglycosides (Sha and Schacht 2000; Campbell et al. 2007) in animals. Protection against cisplatin-induced hearing loss has also been described in humans (Campbell et al. 2009). Data from animal models indicate that D-methionine does not interfere with either the antitumor effect of cisplatin (Cloven et al. 2000) or the antimicrobial action of aminoglycosides (Sha and Schacht 2000).

D-methionine has been effective when administered by intraperitoneal injection, as a pulmonary inhalant, applied directly to the round window, or consumed as an oral suspension (Campbell et al. 1996, 1999, 2007; Korver et al. 2002; Grondin et al. 2013). Having multiple options for different delivery methods can be advantageous in that round window membrane administration may avoid any possible interference with the ototoxic drug's therapeutic effect but precludes any systemic protection from other side effects (e.g., cisplatin-induced peripheral neuropathy). However round window membrane administration may not be practical on a daily basis such as for NIHL or for patients with infectious disease being treated with aminoglycosides who are more likely to have otitis media. Oral administration is generally less expensive, easier, may allow for self-administration and provides the potential of systemic protections. D-methionine is currently in Phase 3 clinical trials (NCT01345474) with the US Department of Defense to prevent permanent NIHL. One Phase 2 clinical trial to prevent cisplatin-induced hearing loss has been completed and further Phase 2 studies for both cisplatin-induced and aminoglycoside-induced hearing loss are planned. Currently oral D-methionine administration is planned for all our clinical trials.

12.5.2 *Ebselen*

A Phase 1 safety study was completed (Lynch and Kil 2009), and now Ebselen is currently in Phase 2 clinical trials to assess potential prevention temporary noise-induced threshold shift (NCT01444846). Several preclinical studies have demonstrated ebselen protection from cisplatin-induced ototoxicity as a single agent (Rybak et al. 2000) or in combination with allopurinol (Lynch and Kil 2005) although not all studies have shown significant ebselen protection from cisplatin-induced ototoxicity (Lorito et al. 2011). Baldew et al. (1990) reported that ebselen did not interfere with cisplatin's antitumor action against MPC 11 plasmacytoma or Prima breast tumor in BALB/c mice. Clinical trials for prevention of cisplatin-induced hearing loss are posted on www.clinicaltrials.gov (see Table 12.2).

Takumida et al. (1999) reported that ebselen also reduced gentamicin-induced ototoxicity in guinea pigs but reportedly no clinical trials for prevention of aminoglycoside-induced hearing loss have been registered on clinicaltrials.gov. Like D-methionine, ebselen can be administered by injection (Rybak et al. 2000) or orally (Lynch and Kil 2005). Currently, all planned clinical trials are for oral administration.

12.5.3 N-*Acetylcysteine*

N-Acetylcysteine has been studied in clinical trials to prevent NIHL but without significant otoprotection (Kramer et al. 2006; Toppila et al. 2002). Lin et al. 2010 reported some protection from temporary threshold shift depending on genetic profile of the workers.

In an early preclinical study, *N*-acetylcysteine was reported to exacerbate aminoglycoside-induced ototoxicity in the guinea pig (Bock et al. 1983). However, in two human studies, *N*-acetylcysteine has been reported to reduce aminoglycoside-induced ototoxicity (Feldman et al. 2007; Tokgoz et al. 2011). Clinical trials for prevention of aminoglycoside-induced ototoxicity are ongoing (see Table 12.2).

Transtympanic injections of *N*-acetylcysteine to prevent cisplatin-induced hearing loss yielded variable results in two clinical trials (Riga et al. 2013; Yoo et al. 2014). Riga et al. (2013) reported statistically significant *N*-acetylcysteine otoprotection for the frequency of 8,000 Hz using the patient's contralateral ear as a control. Yoo et al. (2014) reported that 2 out of 11 patients demonstrated *N*-acetylcysteine protection from cisplatin-induced ototoxicity but group results did not reach statistical significance.

Although *N*-acetylcysteine can be administered either orally (Feldman et al. 2007) or transtympanically (Riga et al. 2013; Yoo et al. 2014) the transtympanic route may be used to avoid any possible interference with the therapeutic action of the ototoxic drug, e.g., cisplatin. Further clinical trials are listed on clinicaltrials.gov (Table 12.2) for both cisplatin and aminoglycoside otoprotection.

12.5.4 Sodium Thiosulfate

Sodium thiosulfate has been studied for decades as a potential otoprotective agent for cisplatin- and carboplatin-induced ototoxicity (Otto et al. 1988; Neuwelt et al. 1996); also see Table 12.2. Clinical trials for otoprotection from platinum-based chemotherapy are ongoing as listed in www.clinicaltrials.gov. Sodium thiosulfate has not been found to prevent NIHL (Pouyatos et al. 2007) or gentamicin-induced hearing loss (Hochman et al. 2006) in preclinical studies, thus sodium thiosulfate clinical trials for otoprotection are currently focusing on chemotherapy otoprotection.

One consideration for sodium thiosulfate as an otoprotective agent is its action as a cisplatin neutralizer when given simultaneously with platinum-based chemotherapeutics (Church et al. 1995; Jones et al. 1991). Consequently, sodium thiosulfate otoprotection protocols have been designed using delay of the sodium thiosulfate administration by several hours after cisplatin administration to provide otoprotection without antitumor interference (Muldoon et al. 2000; Harned et al. 2008). Sodium thiosulfate cisplatin otoprotection has been reported by parenteral administration including injection, intravenous or intra-arterial administration. An oral formulation is available and has been used for other purposes (AlBugami et al. 2013). Several clinical trials for sodium thiosulfate are listed on www.clinicaltrials.org.

12.5.5 Amifostine

Clinical trials with amifostine have not demonstrated significant efficacy in preventing or reducing cisplatin-induced hearing loss, according to a meta-analysis across multiple clinical trials (Duval and Daniel 2012). Currently, it is not recommended for either otoprotection or neuroprotection by the American Society of Clinical Oncology 2008 Clinical Practice Guideline Update Use of Chemotherapy and Radiation Therapy Protectants American Society of Clinical Oncology 2008 clinical practice guideline update: use of chemotherapy and radiation therapy protectants (Hensley et al. 2009). However, it is included in one clinical trial as a secondary end point on clinicaltrials.gov.

12.6 Monitoring for Drug-Induced Hearing Loss

12.6.1 Why and Who Should Monitor?

Formal audiological monitoring during known ototoxic drug administration is vital to detect drug-induced cochlear or vestibular changes clinically. Early detection of ototoxic changes provides the opportunity to consider possibly modifying the course of treatment to reduce these side effects or their progression.

Ototoxicity monitoring is also essential in clinical trials with new drugs that have ototoxic potential and in assessing new otoprotective agents to prevent or treat drug-induced hearing loss. Because ototoxic drugs can first cause subtle hearing changes, patient self-report or informal testing (e.g., tuning forks, watch tick, finger rub) are not sufficient for detecting drug-induced ototoxicity or for pharmacologic protection from drug-induced ototoxicity.

Because intersubject variability for DIHL is high, a priori decisions will need to be clear in the clinical trial design regarding when and if hearing loss changes will result in a subject being discontinued from the clinical trial. Although, no separate formal approved guidelines exist for ototoxicity monitoring with new drugs in clinical trials, the FDA makes recommendations for appropriate ototoxicity monitoring based upon ototoxic potential on a case-by-case basis for each clinical trial. These recommendations include modifications in the protocol if adverse events involving cochlear or vestibular changes occur. Ototoxicity monitoring is also the best means to determine the outcome of clinical trials for new pharmaceutical agents specifically targeted to treat or prevent DIHL.

A successful monitoring program requires the coordination/collaboration/cooperation among the treating physicians, nurses, and the diagnostic audiologists (AAA 2009). Identifying appropriate audiologic support and standardizing the audiologic equipment, personnel, and test procedures across multiple clinical sites, particularly if multiple countries are involved, can be challenging. Hearing healthcare professional organizations, such as the American Speech Language Association (ASHA 1994) and the American Academy of Audiology (AAA 2009), have created guidelines for ototoxicity monitoring. However, no universally accepted standardized protocols currently exist to detect or measure drug-induced ototoxicity as a whole, still less for individual drug classes. At present, each medical facility that actively administers aminoglycoside or antineoplastic therapy is responsible for developing and maintaining their own ototoxicity monitoring program (ASHA 1994). The guidelines developed by the American Speech Language Hearing Association (ASHA 1994) designate the team audiologist with the responsibility for the design and implementation of a comprehensive ototoxicity monitoring program. However, in the case of an FDA-approved clinical trial, all of the clinical trial procedures must be standardized and are part of the overall IND and Institutional Review Board (IRB) documents and procedures cannot be left to individuals at each site. Many clinical audiologists will need to be trained regarding FDA data collection and reporting procedures.

12.6.2 Whom Should Be Monitored?

A major consideration in designing clinical trials to test the efficacy of a pharmacologic agent to prevent DIHL is selection of the patient population. First, a patient population with unavoidable drug-induced ototoxicity of an incidence and degree of hearing loss to test for protection must be identified. These data bases are sometimes not readily available. Other considerations will include accessibility to the subject

pool, other clinical trials they are being recruited to, and whether or not they are able to fully understand and provide written informed consent. In some cases, the patient populations' projected longevity and attrition during a clinical trial may be a factor. The testing time points for audiologic measures will need to be coordinated possibly with patient travel to the clinical area, patient travel costs, and complicated scheduling for other appointments. The clinical trial coordinators will need to work closely with the medical treatment, audiology, and clinical trial team to ensure timely and productive communication between the treating physicians, nurses, and the audiologist. Formal ototoxicity monitoring is presently recommended for patients undergoing treatment with either of the two classes of drugs known to cause severe and permanent hearing loss, the platinum-based antineoplastics (cisplatin and carboplatin) and aminoglycosides (ASHA 1994). Therefore, for pharmacologic protective agents for those two drug classes, it may be possible to coordinate audiologic testing for a protective agent with their usual and customary audiologic care. Currently, monitoring every patient exposed to a potential or low-risk ototoxic agent is neither feasible nor cost-effective clinically, and therefore audiologic testing is not generally a part of their usual and customary clinical care. However, in clinical trials that may include testing to determine if a new drug has even a low incidence of ototoxicity that determination is made on a case-by-case basis for each clinical trial.

The team audiologist(s) should take into consideration the logistics of monitoring hearing thresholds for both adults and children receiving treatment for life-threatening diseases in different environments when designing an ototoxicity monitoring program. For example, in clinical practice, sometimes "bedside" audiologic testing is requested for very ill patients. Studies have shown good test–retest reliability of EHF "bedside" behavioral audiometric threshold responses in hospital wards if appropriate earphones are utilized (Gordon et al. 2005). However, for clinical trials, standardized audiologic test methods including use of a sound treated booth with a patient that can provide reliable data will be needed.

In addition, the audiologist should also develop a comprehensive counseling and rehabilitation program for a patient when ototoxic changes occur. Although the purpose of the clinical trial will be to determine protection from DIHL, even if the protective agent is fully effective, in the placebo group some patients may be expected to develop ototoxic hearing loss during the clinical trial and the patients will require full management of their hearing loss. Until the clinical trial is unblinded, the investigators will not know which subjects are in which arm of the study, but that fact will not alter the need for management of patient hearing loss during the course of the study. The rehabilitation program should include proper education, appropriate fitting of amplification, selection of FM devices or other rehabilitative equipment as needed, or cochlear implantation if necessary. Pre- and post-treatment counseling is an essential part of an ototoxicity monitoring/rehabilitation program as the psychological impact of the combination of a life-threatening disease with the possibility of severe permanent hearing loss can be a devastating situation for a patient and their families.

12.6.2.1 Adults

Baseline Testing

Behavioral audiometric threshold measures are the "gold standard" in monitoring a patient for ototoxic changes. Ideally, a baseline or "pre-treatment" audiological assessment should be as comprehensive as allowable. Some patients may become incapacitated during the course of their drug therapy and thus unable to complete the follow-up test battery. In such cases, objective measures of audiological assessment, such as auditory brainstem response (ABR) testing or otoacoustic emissions (OAE) tests, can be implemented but without baseline data, it is difficult to track changes in these measures. Minimally, an ototoxicity protocol should include both air (from 0.25 to 8 kHz) and bone conduction hearing threshold measures, otoscopic evaluation and tympanometry bilaterally (AAA 2009). This minimal protocol provides threshold monitoring in the conventional speech frequency region to detect changes that have the greatest impact on a patient's ability to understand speech. In addition, bone conduction threshold testing and otoscopic examination along with tympanometry will help detect any middle ear problems at baseline or onset during the drug treatment monitoring period. Otitis media is a common infectious disease among patients who may be immunosuppressed from chemotherapy, especially in the pediatric population (AAA 2009). However, because drug-induced ototoxic changes begin in the ultra-high frequency range first (>8 kHz), it is ideal to include extended high frequency (EHF) monitoring (10–18 kHz) to identify ototoxic changes before they encroach upon the conventional speech frequency range (from 0.25 to 8 kHz). It is recommended that all behavioral audiometric testing be conducted in a sound treated booth/room whenever possible in order to avoid the effects of ambient room noise (i.e., hospital wards) on auditory threshold responses (ASHA 1994).

Follow-up Testing

Scheduled follow-up testing may vary based upon the ototoxic agent and the otoprotective agent used. However, the ASHA (1994) guidelines suggest that clinical follow-up testing should occur: (1) before each administration of an ototoxic agent, (2) at the end of treatment with an ototoxic agent, and (3) at least 6 months after the end of treatment. However, for a clinical trial of a potentially ototoxic drug or for an otoprotective agent, each clinical trial will have to carefully design its testing time points. Regardless of the follow-up schedule, tympanometric measures should always be repeated if any threshold changes occur in order to rule out middle ear dysfunction as the etiology for observed changes in hearing status (AAA 2009). The ASHA guidelines are for early detection of ototoxic change and are frequently used in clinical trials for that purpose. ASHA's (1994) definition of an ototoxic change is: (a) a 20 dB or greater decrease in pure-tone threshold at one frequency, (b) a 10 dB

or greater decrease at two adjacent frequencies, or (c) a loss of response at three consecutive test frequencies in which responses were previously obtained. However, if any of one of these changes occurs, additional scales are needed to grade the degree of adverse events.

A comprehensive baseline ototoxicity monitoring protocol should be easily implemented with relatively healthy adults before the commencement of drug therapy. However, during the course of drug treatment, many patients may become incapacitated and find it difficult to return or undergo subsequent follow-up testing. Furthermore, some patients may become hospitalized and require bedside evaluations in which a portable audiometer can be used if the patient is fully responsive. In these situations, ambient noise levels should always be considered as they may interfere with accurate hearing threshold results, especially for the low frequencies (Thompson and Northern 1981). Investigators need to decide in advance whether bedside data will be included as a part of their clinical trial data. For limited response or unresponsive patients, an objective measure of hearing status with OAEs testing or an ABR test may be necessary. OAE testing is utilized to determine the status of the cochlear OHCs (Kemp 2002) ABR evaluates the status of multiple levels of the afferent auditory brainstem pathways (Bachman and Hall 1998). OAE testing, which can include either transient otoacoustic emissions (TEOAE) or distortion product otoacoustic emissions (DPOAE), is a relatively quick and easy assessment but ABR testing is quite time intensive and limited to frequency analyses of only 1–4 kHz (Mitchell et al. 2004). An advantage of DPOAEs over conventional audiometry is that they appear to detect ototoxic changes before they manifest as hearing loss in the conventional frequency range; however, high frequency audiometry is still more sensitive to ototoxic change, even in children (Knight et al. 2007). One disadvantage of both OAEs and ABRs for evaluation of new otoprotective agents for DIHL is there are no standards for interpreting those data in clinical trials to determine if significant ototoxic change occurred or was prevented.

Auditory steady state response testing (ASSR) has also been investigated as a possible objective testing procedure to assess ultra-high frequency thresholds inaccessible with ABR (10–16 kHz) (Tlumak et al. 2007). However, the feasibility of utilizing ASSR for high frequency ototoxicity monitoring is questionable at this time as ASSR threshold measures have been found to significantly overestimate behavioral threshold measures at these frequency regions (Tlumak et al. 2007). The logistics of trying to incorporate and standardize otoacoustic emissions and electrophysiologic measures into a clinical trial must also be fully considered and budgeted in advance. For example in children, electrophysiologic measures may require extensive time, more complicated scheduling and in many cases sedation and a recovery room. Some of these issues may be reduced in adult subjects and patients; however, we are still left with the challenge of deciding on significant change criteria for electrophysiologic measures.

In clinical trials, while a comprehensive baseline and end of study evaluation are generally implemented, for follow-up testing during the clinical trial, only pure tone air-conduction thresholds are sometimes used with referral to a comprehensive

assessment if significant changes meeting the ASHA criteria occur at any time during the study. The ASHA criteria can be used for both conventional and high frequency audiometric ranges (Campbell et al. 2003).

12.6.2.2 Children

Behavioral audiometric threshold measures are also the "gold standard" for pediatric patients undergoing ototoxicity monitoring (Knight et al. 2007). However, with shorter attention spans and severe illnesses, it may be difficult to obtain reliable and thorough conventional threshold responses in pediatric patients (Chang and Chinosornvatana 2010). Examiners often have to rely on objective measures of threshold estimation with pediatric patients. Ototoxicity monitoring is especially critical in young children as they tend to be at greater risk for ototoxicity than adults (Schell et al. 1989). Even a mild hearing loss has been found to have a substantial impact on the development of emerging speech and language skills (Yoshinaga-Itano and Apuzzo 1998). The loss of high frequency hearing can impair a child's ability to distinguish high frequency speech sounds (s, f, th, k, p, h, sh, ch) which contribute morphological information (i.e., the sound "s" can change the meaning of a word by making it plural) for language development (Stelmachowicz et al. 2004). High frequency hearing loss also makes it difficult to hear speech at a distance and in noisy environments (Stelmachowicz et al. 2004). Although OAE measures have not demonstrated strong correlations with low frequency conventional hearing thresholds in preschool children (Dille et al. 2007), they have shown excellent correlations with high frequency thresholds (Dille et al. 2007) which are typically the frequencies most affected with DIHL.

For clinical trials of ototoxicity or protection from DIHL in children, the testing techniques for the various ages of the children involved must be carefully designed in advance so that the data obtained will be reliable and subject to statistical analyses. Different test procedures may be needed for different age ranges and capabilities.

Follow-up testing in young children in clinical trials may need to be extended to detect progressive hearing loss. Clinically in pediatric cases, post-treatment monitoring is typically extended to longer than 1 year and some ototoxicity monitoring programs continue to monitor up to 3 years post-treatment (Knight et al. 2005). In addition, hearing loss in children that was not detected during the course of treatment has been detected after the completion of ototoxic drug therapy (Bertolini et al. 2004). However, in clinical trials to test for protection from DIHL, early time points may suffice to establish efficacy in the majority of cases.

For the very young, ill or difficult to test child, the use of objective auditory testing procedures (OAEs or ABR) may be necessary (AAA 2009). In those cases, it is essential to establish baseline measures with these tools in order to detect auditory changes throughout drug treatment (AAA 2009). However, as previously mentioned one of the biggest disadvantages of objective testing procedures is that there is no clear consensus regarding which scale or definition constitutes an ototoxic change. Although the International Society of Pediatric Oncology (SIOP) convened in 2010

to create the best version of a pediatric ototoxicity scale (Brock et al. 2012), the scale created is solely based upon the evaluation of auditory threshold results obtained from conventional and extended high frequency behavioral air conduction measures. No scale, to date, has been developed to grade changes in auditory status based upon objective audiometric measures (OAE and ABR) which are common assessment procedures used to monitor fragile pediatric cancer patients unable to undergo comprehensive behavioral assessment (AAA 2009).

12.7 Measuring Ototoxicity

No standard measure of a drug-induced ototoxic change currently exists. The ASHA (1994) criteria are most often used today for DIHL ototoxicity monitoring as these guidelines are sensitive to early ototoxic changes. They are also applicable to monitoring either conventional frequency (250–8,000 Hz) or ultra-high frequency regions (>8,000 Hz). The National Cancer Institute (NCI) also established criteria for ototoxicity as part of the standard NCI assessment which is conducted to monitor adverse events during antineoplastic therapy. This standard assessment measure is known as the common terminology criteria for adverse events (CTCAE) and has undergone three version changes since its initial creation in 1988. Several other independent ototoxicity scales have also been developed and used to define and classify DIHL (Table 12.2) over the last several decades. However, to date, no consensus has been reached on a universally excepted ototoxicity grading measure. Table 12.2 illustrates the variability between the criteria for an "ototoxic change" among several validated and not yet validated grading scales which have been developed and utilized for measuring DIHL ototoxic changes since 1982.

12.7.1 Ototoxicity Grading Scales

Two types of ototoxicity scales have evolved over the last 30 years, the baseline ototoxicity scale and the absolute ototoxicity scale. Baseline ototoxicity scales evaluate hearing changes based upon a pre-treatment baseline auditory evaluation and follow-up evaluations during and after drug treatment. In contrast, absolute ototoxicity scales were developed to grade ototoxic effects in the absence of baseline audiometric measures. Absolute scales are most often utilized in pediatric ototoxicity monitoring as baseline data are often difficult to obtain but because they assume normal hearing at baseline, room for errors in interpretation can exist. The established ASHA criteria (1994) for ototoxic change are still frequently used today as they are very sensitive to early threshold changes, especially for ultra-high frequency responses. However, several other validated and invalidated ototoxicity scales have also been created and used to define and classify DIHL (Table 12.3) over the last several decades. Table 12.2 shows the variability between the criteria

Table 12.3 Ototoxicity scales used to monitor cisplatin ototoxicity

Scale	Reference	Year	Frequency not specified	<4 kHz (dB)	<3 kHz (dB)	<2 kHz (dB)	<1 kHz (dB)
The Khan Scale	Khan et al.	1982	X				
Common terminology criteria for adverse events: CTCAE version. 1 and version. 2	(NCI) National Cancer Institute	1988, 1998	X				
CTCAE version. 3 and version. 4	NCI: National Cancer Institute	2006, 2009	15–20 dB				
ASHA criteria: American Speech/Language Hearing Association	ASHA: American Speech/Language Hearing Association	1994	20 dB or greater at any one frequency; 10 dB or greater at two adjacent frequencies; loss of response at three consecutive frequencies				
WHO: World Health Organization	WHO: World Health Organization	1997		25	25	25	25
Muenster Scale	Schmidt et al.	2007		10–60	10–60	10–60	10–60
Stohr Scale	Stöhr et al.	2005		10–60	10–60	10–60	10–60
Nitz Scale	Nitz et al.	2013		20	20	20	20
Brock “88” Scale[a]	Brock et al.	1988		40	40	40	40
Brock “91” Scale[a]	Brock et al.	1991		40	40	40	
Chang Scale[a]	Chang and Chinosornvatana	2010		20	20	20	
POG: Pediatric Oncology Group Scale	Huang et al.	2002		20–40	20–40	20–40	20–40
FHL: Functional Hearing Loss Scale	Lewis et al.	2009		20	20		
SIOP[a]: International Society of Pediatric Oncology Boston Ototoxicity Grading Scale	Brock et al.	2012		20	20		

[a]Absolute scales

for an "ototoxic change" among several grading scales which have been developed and utilized for measuring cisplatin-induced ototoxic changes since the first published scale in 1982 (Khan et al. 1982).

12.8 Patterns of DIHL

The pattern of hearing loss from drug-induced damage can differ significantly from other patterns of acquired hearing losses, specifically including NIHL. The typical pattern for NIHL reveals a "noise notch" in the audiogram occurring around 4,000 Hz (McBride and Williams 2001). On the other hand, DIHL changes for cisplatin and aminoglycoside antibiotics first occur at EHF regions (~10 kHz) (Singh Chauhan et al. 2011) and progress downward as treatment continues and cumulative dose increases (Strauss et al. 1983). These EHF losses can go undetected when audiological evaluations are confined to the conventional frequency region and do not test frequencies above 8 kHz. However, most ototoxicity monitoring programs now incorporate EHF testing into their monitoring protocols. Unfortunately, ultra-high frequency testing adds significant testing time to the already fairly lengthy testing sessions and very ill patients may not have the stamina to cooperate for a full session. Also not all ototoxic drugs follow the same pattern of hearing loss. For example, difluoromethylornithine (DFMO) may cause a wide variety of audiometric configurations and hearing loss may be reversible (McLaren et al. 2008).

12.9 Monitoring Schedule

12.9.1 Baseline Testing

Baseline audiological testing should be comprehensive and conducted before the first administration of an ototoxic medication. For antineoplastics, baseline testing should be performed before treatment. However, for aminoglycosides, testing may be conducted within the first 72 h after first administration as it has been shown that cochleotoxic changes usually do not manifest until that time (AAA 2009). Scheduling and conducting audiological baseline testing can be challenging with individuals requiring aminoglycoside therapy as these drugs are most often given on an emergency basis as opposed to the planned administration of antineoplastics.

12.9.2 Follow-up Testing

Periodic audiologic re-evaluations should occur throughout the course of all ototoxic drug treatment. There are currently no universal guidelines for audiologic monitoring for specific ototoxic medications. However, for those taking aminoglycosides,

clinical guidelines (AAA 2009) recommend weekly or at least biweekly reassessments during the course of drug treatment. Follow-up treatment assessments are also recommended a few months after the drug completion due to the potential for delayed hearing loss with aminoglycoside therapy (AAA 2009). On the other hand, the ASHA (1994) guidelines are less specific but recommend that the audiologist implement monitoring intervals during drug treatment which are optimum to detect ototoxic changes based upon the ototoxic medication administered. However, ASHA (1994) does recommend immediate post-treatment assessments for all ototoxic medications. Each clinical trial will need to carefully design its time points for audiologic assessments based on the time course of the anticipated hearing loss anticipated for the drug agent in the specific population under investigation.

12.10 Drug-Induced Vestibular Toxicity

12.10.1 Vestibular Monitoring

Monitoring for drug-induced vestibular changes, like cochlear changes, has it challenges. Like DIHL, there are no universal standards for evaluating and grading drug-induced vestibular changes and a thorough monitoring protocol usually requires a battery of vestibular assessments which may be overwhelming for very ill patients. The objective vestibular assessments, such as the vestibular evoked myogenic potential (VEMP), head thrust testing, visual acuity testing, and horizontal head impulse testing can be performed at bedside, but other objective tests (i.e., the rotary chair and vestibular autorotation (VAT)) must be conducted in the laboratory and require ambulation on the part of the patient (Black and Pesznecker 2007). In addition, subjective evaluation can be made using the Dizziness Handicap Inventory (DHI), a validated 25-item questionnaire to assess the impact of a patient's vestibular symptoms on quality of life (Handelsman 2007). However, most patients with aminoglycoside-induced vestibulotoxicity will acquire bilateral deficits and most often will not display the overt vestibular symptoms of vertigo and nystagmus seen in unilateral vestibular dysfunction (Minor 1998).

Most patients who would benefit from vestibular monitoring are usually battling a life-threatening disease and will be quite fragile and possibly nonambulatory during ototoxic drug treatment. A significant portion of these patients may also be experiencing nausea and vomiting from drug treatments and cannot tolerate routine vestibular assessments which exacerbate these conditions (Black and Pesznecker 2007). Sedatives are also routinely used in the care of these patients and can mask subjective vestibular symptoms during the course of treatment with symptoms only manifesting once treatment has been discontinued and vestibular damage has already occurred (Black and Pesznecker 2007).

As with DIHL, the development of a vestibular monitoring program should be the responsibility of the diagnostic team audiologist. No consensus has been established as to which vestibular test procedures should be utilized to best monitor drug-induced

vestibulotoxicity (Handelsman 2007). As with DIHL monitoring, a test battery approach offers the most comprehensive analysis of vestibular changes during drug treatment. As for the monitoring schedule, it is recommended that vestibulotoxic monitoring include baseline assessment measures, serial monitoring throughout drug treatment and assessment at the end of treatment (Handelsman 2007).

For clinical trials, it must first be determined if vestibulotoxicity is the focus of the clinical trial or if the clinical trial of a new drug to prevent DIHL focuses on hearing alone. If the protective agent focuses on hearing preservation only, and determination of whether or not it influences vestibular status is secondary or exploratory, the DHI may provide a quick, reliable method for monitoring (Campbell et al. 2003).

12.10.2 Vestibular Management

Counseling is an important part of a vestibular monitoring program as this side effect alone can leave patients permanently disabled and unable to resume normal activities (Black and Pesznecker 2007; Handelsman 2007). Vestibular rehabilitation is recommended for all those who experience drug-induced vestibulotoxicity. Patients who acquire unilateral vestibular losses can usually benefit from adaptation exercises to improve their vestibular reflexes. However, those who acquire bilateral losses must learn substitution strategies with their other senses in order to stabilize their gaze and help them with ambulation (Minor 1998). Again, currently no clear guidelines for assessing the vestibular efficacy of an otoprotective agent in clinical trials exist, although some clinical trials have used the DHI to assess drug-induced ototoxicity (Campbell et al. 2003).

12.11 Future Outlook

The past decades have yielded marked improvements in our ability to assess ototoxic changes across a variety of patient population. Further a number of new scales for early detection of ototoxicity, and grading ototoxicity have allowed better quantification of the data and comparison across studies. The wide variety of scales being used can have the advantage of allowing the investigator to select the measure most applicable to their study population, the lack of agreement on which scales to use in similar populations renders comparisons across studies problematic. Further not all drugs affect hearing in an identical manner or across the same time course. As otoprotective agents progress through multiple clinical trials, we will need to arrive at some method for comparing the relative efficacy of these agents. Further, we need better measures for the assessment of drug-induced vestibular function.

Nonetheless, the progress has been remarkable and the current and future clinical trials for otoprotective agents against various drug-induced ototoxicities suggest

that in the future we may be able to reduce or eliminate ototoxic hearing loss in a variety of patient populations. Preserving the hearing in these patients could markedly improve their quality of life.

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Part VI
Oxidative Stress and Age-Related Hearing Loss

Chapter 13
Age-Related Hearing Loss: Mitochondrial Biochemical Pathways and Molecular Targets

Mi-Jung Kim, Karessa White, Logan Walker, Chul Han, and Shinichi Someya

13.1 Introduction: Mitochondrial Dysfunction as a Cause of Hearing Loss

The mammalian mitochondrial genome consists of 37 genes encoding 2 ribosomal RNAs (rRNAs), 22 transfer RNAs (tRNAs), and 13 polypeptides that are all involved in oxidative phosphorylation system (Wallace 2005; Montoya et al. 2006). Since these mitochondrial genes play an essential role in energy metabolism in the inner ear, mitochondrial DNA (mtDNA) mutations and associated mitochondrial dysfunction have been hypothesized to contribute to hearing loss (Kujoth et al. 2005; Someya and Prolla 2010; Kokotas et al. 2007; Someya et al. 2008). Direct evidence for mitochondrial dysfunction in hearing loss comes from the observations that accumulation of mtDNA mutations leads to premature aging phenotypes such as reduced lifespan, osteoporosis, and early onset of hearing loss in mice carrying a mutator allele of the mtDNA polymerase gamma (*POLG*), the only DNA polymerase that is active in the mitochondria and that can proofread and replicate mtDNA (Someya and Prolla 2010; Kujoth et al. 2007; Someya et al. 2008; Trifunovic et al. 2004). Several mutations in *POLG* have been identified as a cause of human disorders such as Alper's syndrome and deafness (Someya and Prolla 2010; Kujoth et al. 2007; Mancuso et al. 2004; Nguyen et al. 2005). Specific point mutations in mtDNA also contribute to mitochondrial disorders in humans such as mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS) and myoclonic epilepsy and ragged red fibers (MERRF), the symptoms of which include hearing loss (Chinnery et al. 2000; Fischel-Ghodsian 2003;

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Kokotas et al. 2007). Furthermore, more than 100 different deletions of mtDNA have been associated with mitochondrial disorders, and of these, some mtDNA deletions cause Kearns-Sayre syndrome which also involves hearing loss (Chinnery et al. 2000; Fischel-Ghodsian 2003; Kokotas et al. 2007). Collectively, these reports support the idea that mitochondrial defects or dysfunction play a key role in the development of hearing loss.

Mitochondria are a major source of reactive oxygen species (ROS) and a major site of ROS-induced oxidative damage (Balaban et al. 2005; Wallace 2005; Shigenaga et al. 1994; Finkel and Holbrook 2000). It is estimated that ~90 % of intracellular ROS are continuously generated as a by-product of mitochondrial respiration metabolism during the generation of ATP (Balaban et al. 2005). The production of superoxide ($O_2^{\bullet -}$), one of the major ROS, is thought to occur at Complex I (NADH dehydrogenase), Complex II (succinate dehydrogenase), or Complex III (coenzyme Q-cytochrome c reductase) of the mitochondrial electron transport chain (Fig. 13.1) (West et al. 2011). Under normal metabolic conditions, Complex I and Complex III are thought to be the main sites of superoxide production (Balaban et al. 2005; West et al. 2011). Superoxide from Complex I and Complex II is released into the matrix, whereas superoxide from Complex III is released into the intermembrane space or matrix (West et al. 2011). It is also estimated that ~10 % of

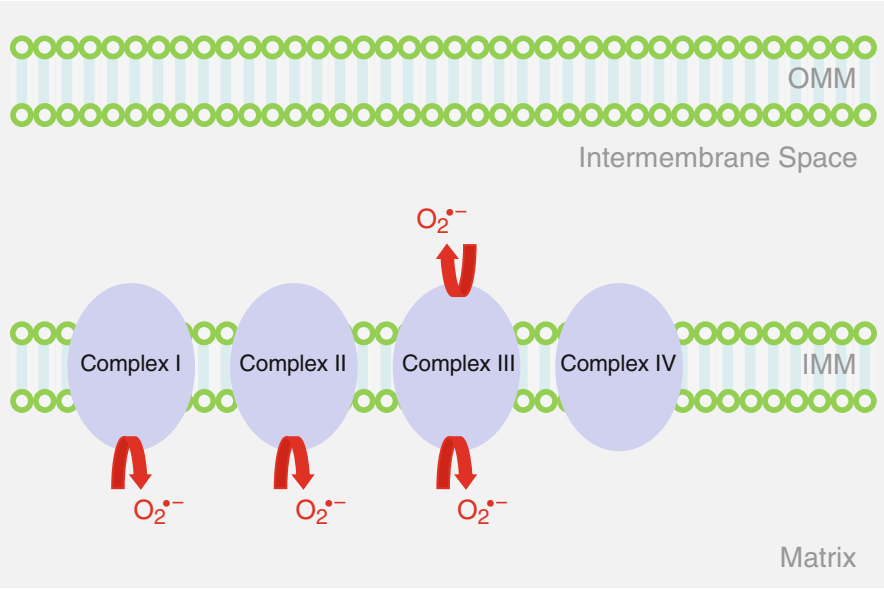

Fig. 13.1 Superoxide production in the mitochondrial electron transport chain. The production of superoxide ($O_2^{\bullet -}$) is thought to occur at Complex I (NADH dehydrogenase), Complex II (succinate dehydrogenase), or Complex III (coenzyme Q-cytochrome c reductase), but under normal metabolic conditions, Complex III is thought to be the main sites of superoxide production. Superoxide from Complex I and Complex II is released into the matrix, whereas superoxide from Complex III is released into the intermembrane space or matrix. OMM=outer mitochondrial membrane, IMM=inner mitochondrial membrane)

Table 13.1 Mitochondrial antioxidant defense enzymes

Enzyme	Enzymatic reaction
SOD2	$2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$
GPX1	$H_2O_2 + 2GSH \rightarrow GSSG + 2H_2O$
GSR	$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+$
PRDX3	$H_2O_2 + TXN2_{red} \rightarrow TXN2_{oxi} + 2H_2O$
TXNRD2	$TXN2_{oxi} + NADPH + H^+ \rightarrow TXN2_{red} + NADP^+$

intracellular ROS are generated within the plasma membranes and peroxisomes. A large body of evidence indicates that ROS play a role in aging and a variety of age-related disease (Balaban et al. 2005; Wallace 2005; Harman 1956; Finkel and Holbrook 2000; Shigenaga et al. 1994). This hypothesis is supported by the observations that overexpression of the mitochondrial antioxidant enzyme SOD2 (superoxide dismutase 2) significantly increases the lifespan of flies (Sun et al. 2002), while overexpression of a mitochondrially targeted catalase transgene (MCAT) results in reduced age-related pathology in the heart and increases lifespan in mice (Schriner et al. 2005). Moreover, supplementation with the mitochondrial antioxidant alpha-lipoic acid or N-acetylcysteine reduces oxidative damage in the brain of aged rodents (Palaniappan and Dai 2007; Banaclocha 2001).

Mitochondria are protected against ROS by an interacting network of antioxidant enzymes (Table 13.1) (Balaban et al. 2005; Halliwell and Gutteridge 2007; Finkel and Holbrook 2000; West et al. 2011). SOD2 converts superoxide into hydrogen peroxide, which in turn is decomposed to water by two major mitochondrial antioxidant defense systems: the glutathione and thioredoxin systems (Halliwell and Gutteridge 2007; Mari et al. 2009; Ribas et al. 2014; Aon et al. 2012). There are three major players in the mitochondrial glutathione system: glutathione, glutathione peroxidase 1 (GPX1), and glutathione reductase (GSR). Glutathione acts as the major antioxidant in cells. When glutathione is in a reduced state or an active form, it interacts with GPX1 to decompose hydrogen peroxide into water. NADPH-dependent GSR then reduces oxidized glutathione (GSSG) to reduced glutathione (GSH). There are three major players in the mitochondrial thioredoxin system: thioredoxin 2 (TXN2), peroxiredoxin 3 (PRDX3), and thioredoxin reductase 2 (TXNRD2). When TXN2 is in a reduced state, it interacts with PRDX3 to decompose hydrogen peroxide into water. NADPH-dependent TXNRD2 then regenerates reduced TXN2 ($TXN2_{red}$) from oxidized TXN2 ($TXN2_{oxi}$). These antioxidant defense systems work with each other to protect key mitochondrial components such as mtDNA, proteins, and membranes from ROS-induced oxidative damage (Evans and Halliwell 1999). It is thought that the mitochondrial antioxidant defense systems do not keep pace with the age-related increase in mitochondrial ROS production, and that during aging, the balance between the mitochondrial antioxidant defense and ROS production shifts progressively toward a more pro-oxidant state (Rebrin and Sohal 2008). Therefore, the balance between ROS production and mitochondrial antioxidant defense capacity may determine the degree of ROS-induced oxidative damage and associated mitochondrial dysfunction in the inner ear sensory hair cells or spiral ganglion neurons, which require larger amounts of

energy to process sounds. In this chapter, we summarize what has been learned about the mitochondrial biochemical pathways and/or targets of age-related hearing loss (AHL), and examine the link between aging, mitochondrial dysfunction, and hearing loss in rodents and humans.

13.2 Molecular Targets of AHL Involved in the Maintenance of Mitochondrial Function

13.2.1 Citrate Synthase

Citrate synthase (CS) is the first and rate-limiting enzyme of the tricarboxylic acid (TCA) cycle in the mitochondria (Johnson et al. 2012: Raimundo et al. 2011). CS is encoded by a single nuclear gene and is localized in the mitochondrial matrix where it functions as the first step of the TCA cycle, producing citric acid from acetyl-CoA and oxaloacetate (Fig. 13.2). Hence, CS plays a critical role in energy metabolism. Johnson et al. mapped an *ahl4* locus on Chromosome 10 that contributes to AHL of the A/J mouse strain (Johnson et al. 2012), and found that a CS mutation underlies *ahl4*-related

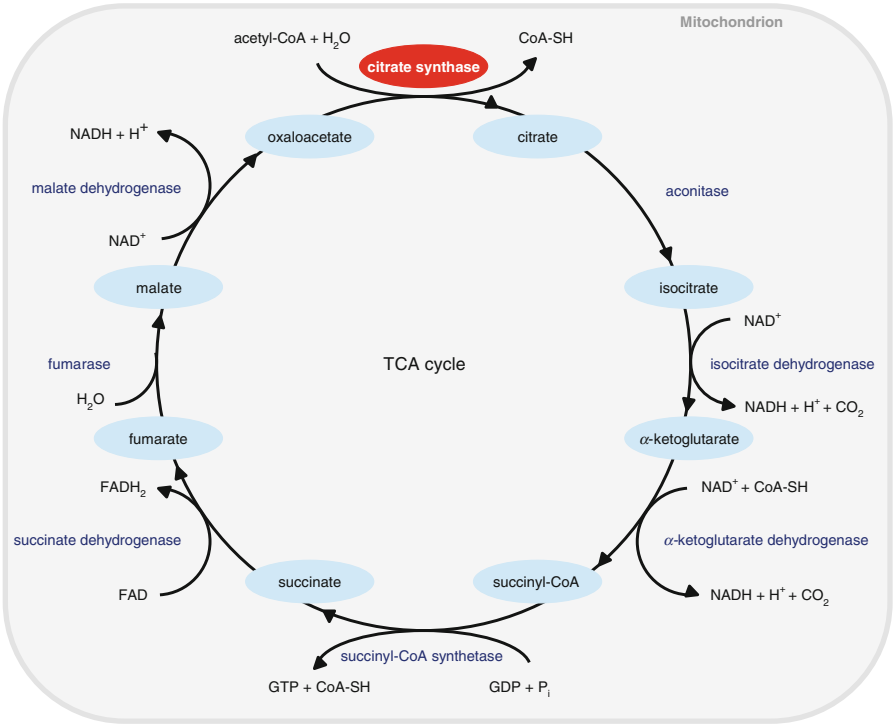

Fig. 13.2 TCA cycle. Citrate synthase (CS) is the first and rate-limiting enzyme of the TCA cycle in the mitochondria

hearing loss in the A/J strain. These A/J mice display early onset and rapid progression of hearing loss. The authors sequenced the entire genomes of A/J and four strains that lack an *ahl4* phenotype, and found that the rs29358506 single nucleotide polymorphism (SNP), located in exon 3 of CS, is the only exon or splice site variant within the 5.5 megabases (Mb) candidate region that is unique to the A/J strain, indicating that CS is the gene responsible for *ahl4*. Consistent with these results, A/J mice display poor performance in endurance exercise such as having the shortest treadmill duration time among ten inbred mouse strains, including the C57BL/6 strain (Lightfoot et al. 2001). Another study has also shown poor treadmill performance in A/J mice, estimating the endurance capacity of BALB/c mice to be 217 % greater than that of A/J mice (Haramizu et al. 2009). Collectively, these results support the importance of CS in energy metabolism and are consistent with the suggestion that a decline in CS activity and resulting decreased ATP production in the inner ear tissues may lead to mitochondria dysfunction and associated cochlear cell death.

13.2.2 Uncoupling Protein 2

Uncoupling protein 2 (UCP2) is a member of the mitochondrial anion carrier protein family, which facilitates the transfer of anions from the inner to the outer mitochondrial membrane and the return transfer of protons from the outer to the inner mitochondrial membrane (Fig. 13.3) (Sugiura et al. 2010; Mattiasson et al. 2003). UCPs also reduce the mitochondrial membrane potential in mammalian cells. UCP2 is expressed in multiple tissues, including skeletal muscles and central nervous system and is thought to play a role in thermogenesis, obesity, and diabetes. A neuroprotective role for UCP2 was indicated by the finding that brain damage following experimental stroke was reduced in mice overexpressing human *UCP2* (Mattiasson et al. 2003). Moreover, *UCP2*, *UCP3*, and *UCP4* mRNAs were found to be expressed in the vestibular and spiral ganglions in the inner ear, while *UCP3* mRNA expression was undetectable in the brain of rats (Kitahara et al. 2004). A human epidemiological study in a Japanese population discovered an association between the *UCP1* A-3826G polymorphism (rs1800592) and obesity (Sugiura et al. 2010). Furthermore, Sugiura et al. investigated the association between *UCP1* and *UCP2* gene polymorphisms and hearing impairment in middle-aged and elderly males and females in Japan (Sugiura et al. 2010). Detailed questionnaires, pure-tone audiometry measurements, and *UCP1* A-3826G and *UCP2* Ala55Val polymorphisms were examined in 1,547 subjects between the ages of 40 and 79 years. Using generalized estimating equations, associations between hearing impairment and the gene polymorphisms in *UCP1* and *UCP2* with age, sex, history of occupational noise exposure, and body mass index were analyzed under dominant, recessive, and additive models. Sugiura et al. found that *UCP2* Ala55Val polymorphisms exhibited a significant association with AHL in the Japanese population. Together, these reports suggest that a decline in mitochondrial UCP2 activity may accelerate the progression of AHL in humans.

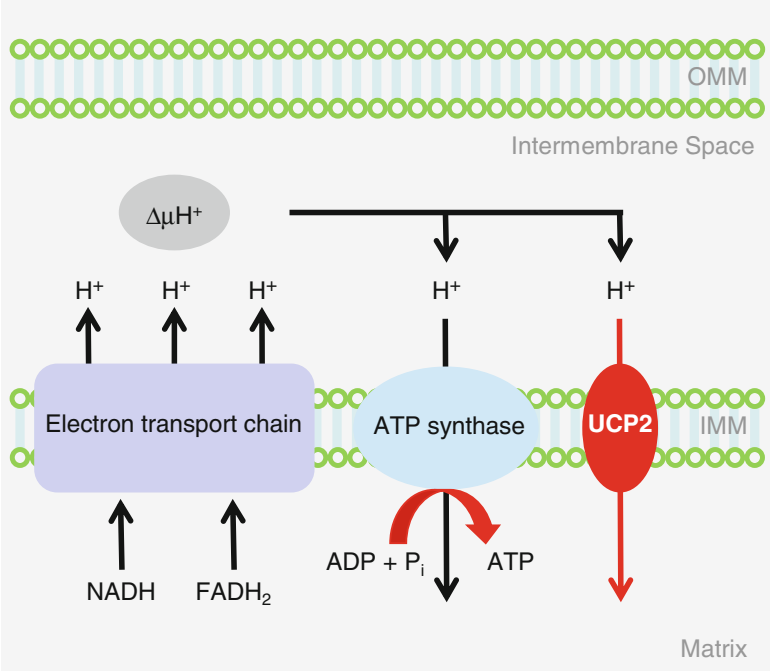

Fig. 13.3 Mitochondrial uncoupling. Uncoupling protein 2 (UCP2) is a member of the mitochondrial anion carrier protein family, which facilitates the transfer of anions from the inner to the outer mitochondrial membrane and the return transfer of protons from the outer to the inner mitochondrial membrane. OMM=outer mitochondrial membrane, IMM=inner mitochondrial membrane

13.2.3 Mitochondrial DNA Polymerase Gamma

mtDNA *POLG* is the only DNA polymerase that is active in the mitochondria and that can proofread and replicate mtDNA (Fig. 13.4) (Kujoth et al. 2007). *Polg* knockin mice were created by introducing a two-base substitution, which results in a defect in mtDNA proofreading ability (Kujoth et al. 2005; Trifunovic et al. 2004). Young *Polg* mutator mice were indistinguishable from wild-type (WT) littermates, but these mutator mice displayed a variety of premature aging phenotypes such as kyphosis and alopecia by 9 months of age. More importantly, *Polg* mutator mice displayed a reduced life span: the median survival of the *Polg* mutator mice was 416 days, while the median life span of WT mice was >850 days. Age-related weight loss, a common feature of the elderly, was noted from 24 weeks of age in these animals. Nine months old mutator mice also displayed reduced bone mineral density, a common feature of age-related osteoporosis and age-related loss of skeletal muscle, a common feature of sarcopenia. Auditory brainstem response (ABR) hearing tests were conducted in these animals: young *Polg* mutator mice displayed normal hearing compared to age-matched WT mice: however, by 9–10 months of age, *Polg* mutator mice displayed a significant elevation of ABR thresholds at 4, 8, and 16 kHz

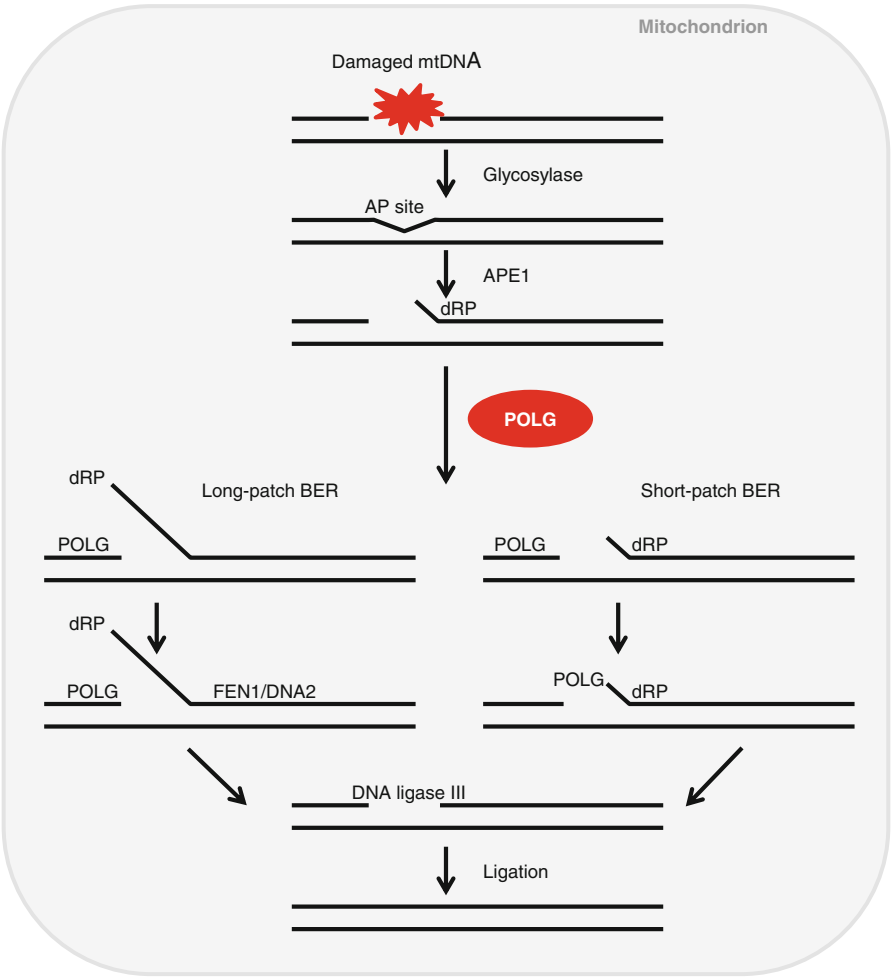

Fig. 13.4 Mitochondrial DNA repair. Mitochondrial DNA polymerase gamma (POLG) is the only DNA polymerase that is active in the mitochondria and that can proofread/repair and replicate mtDNA. AP site (apurinic/apyrimidinic (AP) site), APE1 (AP endonuclease 1), dRP (5' 2-deoxyribose 5-phosphate), BER (base excision repair), FEN1 (flap endonuclease 1), DNA2 (DNA replication ATP-dependent helicase/nuclease)

(Kujoth et al. 2005; Someya et al. 2008; Niu et al. 2007). Examination of the histology of the basal cochlear region confirmed that 9–10-month-old *Polg* mutator mice displayed severe loss of spiral ganglion neurons and hair cells (Kujoth et al. 2007; Someya et al. 2008; Niu et al. 2007). These animal study results were consistent with the observation that a defect in the human *POLG* gene causes Alper's syndrome that is associated with both mitochondrial dysfunction and hearing loss (Kujoth et al. 2007; Someya and Prolla 2010; Mancuso et al. 2004). Together, these reports suggest that a decline in POLG activity in the mitochondria causes mitochondrial dysfunction and associated cochlear cell loss, leading to the progression of AHL.

13.2.4 Glutathione Peroxidase 1

Glutathione peroxidase 1 (GPX1) plays an important role in mitochondrial antioxidant defense by decomposing hydrogen peroxide into water (Fig. 13.5) (Halliwell and Gutteridge 2007; Mari et al. 2009). A previous study has shown that cochlear ROS levels were elevated following acute noise exposure in C57BL/6 mice (Ohlemiller et al. 1999). Consistent with this report, mice lacking superoxide dismutase 1 (*Sod1*) displayed increased hair cell loss in the cochlea compared to WT mice (McFadden et al. 1999). Oxidative protein damage also increases with age in the cochleae of CBA/J mice (Jiang et al. 2007), while oxidative nuclear DNA damage increases with age in the cochlea of C57BL/6 mice (Someya et al. 2009). Ohlemiller et al. (2000) investigated whether *Gpx1* deficiency increases susceptibility to noise-induced hearing loss (NIHL) in mice and found that $Gpx1^{-/-}$ mice showed significantly greater ABR threshold elevation after noise exposure compared to WT mice. Noise-exposed $Gpx1^{-/-}$ mice also showed more sensory hair cell loss compared to WT mice. These results suggest that a decline in GPX1 activity in the mitochondria may lead to increased levels of ROS, which in turn results in mitochondrial dysfunction, leading to cochlear cell loss and associated hearing loss.

13.2.5 Catalase

Catalase (CAT) converts hydrogen peroxide into water and oxygen, and is one of the most efficient enzymes found in cells (Fig. 13.6) (Halliwell and Gutteridge 2007): Each catalase molecule can decompose millions of hydrogen peroxide molecules every second, indicating the importance of this antioxidant enzyme in protecting the

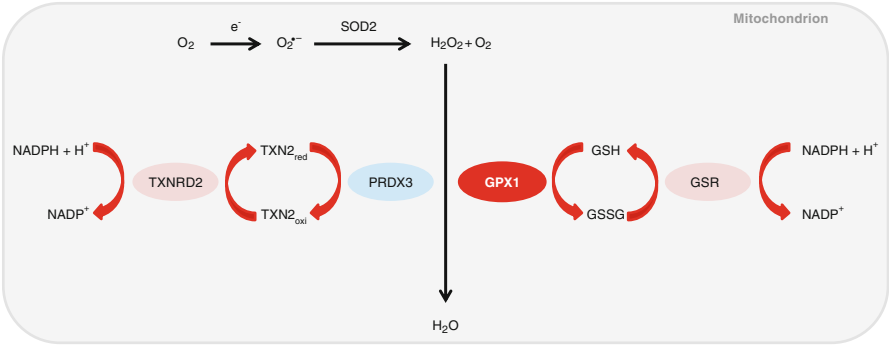

Fig. 13.5 Mitochondrial antioxidant defense system. Glutathione peroxidase 1 (GPX1) plays an important role in mitochondrial antioxidant defense by decomposing hydrogen peroxide into water. TXNRD2=thioredoxin reductase 2, PRDX3=peroxiredoxin 3, GPX1=glutathione peroxidase 1

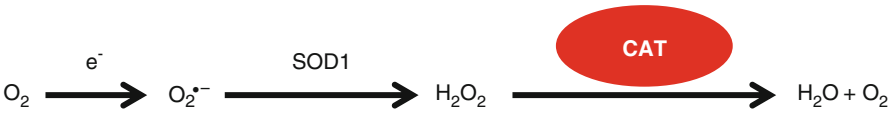

Fig. 13.6 Peroxisomal/cytosolic antioxidant defense system. Catalase (CAT) is localized in the peroxisome or cytosol and converts hydrogen peroxide into water and oxygen in the cells

cells against ROS. Catalase is usually localized in the peroxisomes, although catalase expression was also observed in the cytosol and mitochondria. Schriner et al. generated mice overexpressing human catalase targeted to the peroxisome (PCAT), nucleus (NCAT), or mitochondria (MCAT) and found that overexpression of catalase in the mitochondria extended both median and maximum life span in both males and females, while overexpression of catalase in the nuclei did not show a significant extension of median nor maximum life span (Schriner et al. 2005). PCAT animals showed a slight extension of median life span. MCAT mice also displayed increased CAT activities in the heart, skeletal muscle, and brain, reduced oxidative DNA damage, and decreased hydrogen peroxide levels in the heart. In agreement with these reports, young MCAT mice display normal hearing compared to age-matched WT mice: however, middle-aged MCAT mice display significantly lower ABR thresholds at 8, 16, and 32 kHz than those of middle-aged WT mice. Furthermore, MCAT mice display reduced oxidative DNA damage and loss of spiral ganglion neurons and hair cells in the cochlea (Someya et al. 2009). Importantly, in humans, CAT activity was significantly higher in the red blood cells of centenarians compared to young healthy adults (Kłapcińska et al. 2000). Collectively, these results suggest that enhancing CAT activity and/or reducing hydrogen peroxide levels in cochlear mitochondria may slow the development of age-related cochlear degeneration and hearing loss.

13.2.6 BCL-2-Antagonist/Killer

It is well-documented that mitochondria play a major role in apoptosis which is regulated by BCL-2 (b-cell lymphoma 2) family members (Mattson 2000; Youle and Strasser 2008; Lindsten et al. 2000). Of the BCL-2 family members, the pro-apoptotic proteins BCL-2 antagonist/killer (BAK) and BCL-2-associated X (BAX) play a central role in promoting mitochondrial-mediated apoptosis. These proapoptotic proteins promote permeabilization of the outer mitochondrial membrane, leading to caspase activation and cell death (Fig. 13.7). Lindsten et al. generated *Bak*-deficient mice and found that homozygous $Bak^{-/-}$ mice are fertile and do not display any developmental abnormalities, suggesting that the role of BAK in development may be redundant with that of other proapoptotic BCL-2 family members such as BAX (Lindsten et al. 2000). We investigated whether BAX and BAK may function in a redundant manner in the auditory system and found no significant

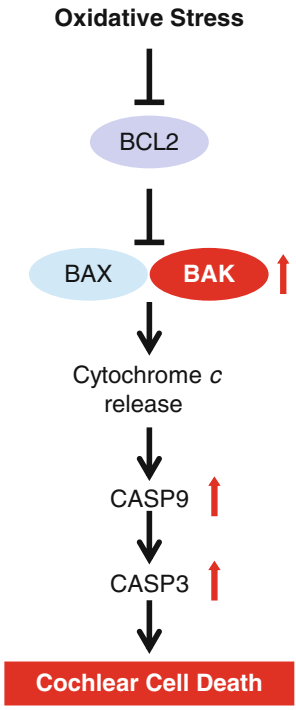

Fig. 13.7 Mitochondrial apoptosis. BCL-2-antagonist/killer (BAK) promotes permeabilization of the outer mitochondrial membrane, leading to caspase activation and cell death. CASP9=caspase-9, CASP3=caspase-3

differences between middle-aged WT and *Bax*$^{-/-}$ mice; both had similar hearing loss indicated by higher ABR thresholds and the lower numbers of hair cells and spiral ganglion neurons typically seen in middle-aged C57BL/6 mice (Someya et al. 2009). In contrast, middle-aged *Bak*$^{-/-}$ mice displayed significantly lower ABR thresholds at 8, 16, and 32 kHz and fewer losses of spiral ganglion neurons and hair cells than those of age-matched WT. Moreover, TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) staining revealed that the levels of apoptotic nuclear fragmentation markers were significantly reduced in the cochlea of middle-aged *Bak*$^{-/-}$ mice compared to age-matched WT mice. Therefore, a decline in BAK-mediated mitochondrial apoptosis may prevent mitochondrial dysfunction, which in turn prevents cochlear cell death, and the progression of hearing loss during aging.

13.3 Conclusions

We have reviewed what has been learned about the mitochondrial biochemical pathways associated with AHL, including the TCA cycle, mitochondrial uncoupling, mtDNA repair, mitochondrial antioxidant defense, and mitochondrial apoptosis pathways (Fig. 13.8), and examined the link between aging, mitochondrial dysfunction, and hearing loss in rodents and humans. Accumulating evidence indicates that mitochondrial dysfunction plays a key role in the development of age-related

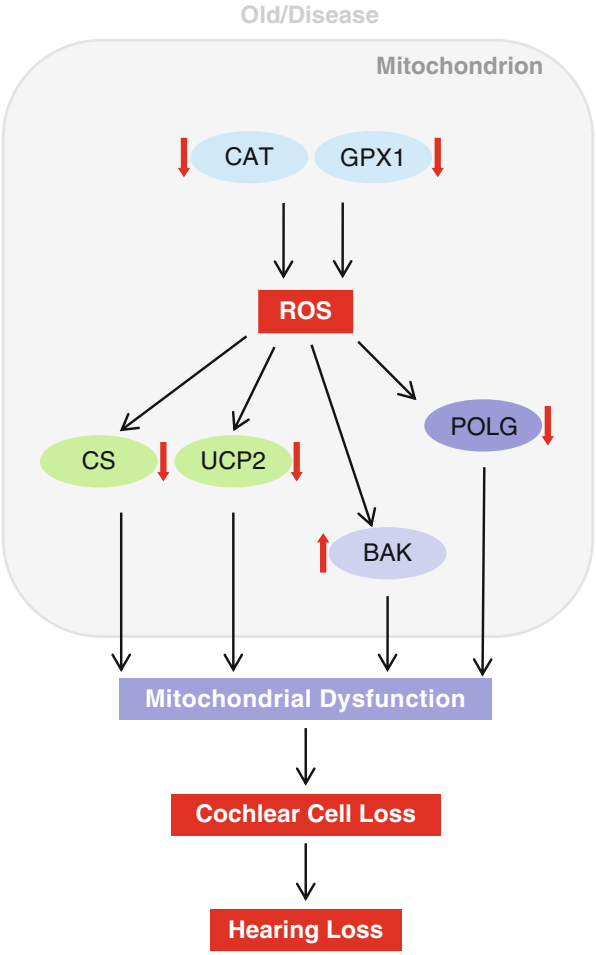

Fig. 13.8 Models for mitochondrial CS, UCP2, POLG, GPX1, CAT, and BAK in mitochondrial dysfunction. A decline in the activities of CS, UCP2, POLG, GPX1, CAT, or an increase in BAK activity may promote mitochondrial dysfunction, which in turn leads to cochlear cell loss and associated hearing loss

neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS) (Finkel and Holbrook 2000; Mari et al. 2009; Mattiasson et al. 2003). AD is characterized by progressive impairment of cognition and emotional disturbances that are associated with synaptic degeneration and loss of neurons (Mattiasson et al. 2003). Increased oxidative damage has been found in the brain of AD patients (Haass and Selkoe 2007), while loss of total glutathione as well as altered glutathione redox status has been observed in the brain of AD individuals (Mari et al. 2009; Boyd-Kimball et al. 2005). PD is characterized by profound motor dysfunction due to degeneration of dopamine neurons in the substantia nigra of the brain (Mattiasson et al. 2003). The early biochemical features of

PD include increased mitochondrial dysfunction, oxidative damage, and loss of total glutathione in the dopamine neurons (Perry and Yong 1986). ALS is characterized by progressive paralysis due to degeneration of motor neurons in the spinal cord, and mutations in the *SOD1* gene are responsible for some inherited cases of ALS (Mattiasson et al. 2003; Gurney et al. 1994; Wong et al. 1995). Alper's syndrome, caused by mutations in *POLG*, is a progressive degenerative disease of the central nervous system that is associated with progressive sclerosing poliodystrophy and deafness (Someya and Prolla 2010; Kujoth et al. 2007; Mancuso et al. 2004). Individuals with Alper's syndrome have fewer copies of mtDNA in the tissues affected by the disease. This mtDNA depletion leads to impaired oxidative phosphorylation and a decrease in cellular energy, which in turn affect postmitotic tissues such as brain, muscle, and inner ear tissue. It is thought that brain, muscle, and inner ear tissues are most affected because those tissues require larger amounts of energy, are more dependent on oxidative phosphorylation for energy, and damaged cells in these non-regenerative tissues are not generally replaced by new cells, resulting in the variety of symptoms of Alper's syndrome. Therefore, we speculate that a decline in the activities of mitochondrial enzymes or proteins such as *POLG*, *CS*, or *UCP2* could play a key role in mitochondrial dysfunction, which in turn leads to development of human age-related progressive hearing loss (Fig. 13.8).

If oxidative stress plays a causative role in mitochondrial dysfunction in the inner ear, then a defect in antioxidant enzyme activities may result in decreased protection of the mtDNA, proteins, enzymes, and membranes against ROS-induced oxidative damage. In agreement with this hypothesis, overexpression of catalase targeted to the mitochondria reduces oxidative nuclear DNA damage in the cochlea and slows the development of AHL (Someya et al. 2009), while deficiency of *Gpx1* increases susceptibility to noise-induced loss of sensory hair cells and hearing in mice (Ohlemiller et al. 2000). Consistent with this report, outer hair cells are the primary lesion from loud noise exposure (Liu and Yan 2007; Ohlemiller et al. 1999), while ROS are generated in the cochlea of chinchillas and mice after exposure to high-intensity noise (Jacono et al. 1998; Ohlemiller et al. 1999). In humans, an association between AHL and the glutathione enzyme family members *GSTT1* (glutathione *S*-transferase theta 1) and *GSTM1* (glutathione *S*-transferase mu 1) polymorphisms has been detected in the Finish population (Van Eyken et al. 2007). A recent study also showed a significant association between AHL and the *GSTM1* and the *GSTT1* polymorphisms in the Hispanic population (Bared et al. 2010). In a Chinese noise-exposed population, an association between NIHL and *SOD1* polymorphisms has been detected (Liu et al. 2010). Furthermore, longer employment duration was found to be associated with poorer hearing levels and lower catalase activity in the plasma in Turkish textile worker population (Yildirim et al. 2007), while an association between noise exposure levels and two *CAT* polymorphisms has been detected in Swedish and Polish noise-exposed laborers (Konings et al. 2007). Therefore, we speculate that noise-induced oxidative damage may cause a decline or defect in the activities of cochlear antioxidant defense enzymes such as SOD1, SOD2, CAT, and GPX1. This in turn results in mitochondrial dysfunction, leading to cochlear cell loss and associated hearing loss (Fig. 13.8).

Currently, there is no treatment or intervention for human AHL. However, in animals, both reduced caloric intake and decreased nutrient-sensing pathway activity are known to slow the rate of aging and lower the incidence of age-related diseases, including neurodegenerative diseases and hearing loss (Fontana et al. 2010). Our previous study has shown that under a long-term calorie-restricted condition, mitochondrial Sirt3, a member of the sirtuin family, mediates the reduction of oxidative damage in the cochlea and slows the development of AHL by enhancing the activity of glutathione reductase which converts oxidized glutathione to reduced glutathione, the active form of glutathione in the mitochondria (Someya et al. 2010). As discussed earlier, *Gpx1* deficiency increases susceptibility to noise-induced loss of sensory hair cells and hearing in mice (Ohlemiller et al. 2000), while in humans, an association between AHL and *GSTT1* and *GSTM1* polymorphisms has been detected in the Finish and Hispanic population (Van Eyken et al. 2007; Bared et al. 2010). Therefore, we speculate that regular dietary restriction may enhance the mitochondrial glutathione antioxidant defense pathway, which in turn maintains mitochondrial function, protects cochlear cells against oxidative damage, and slows the progression of AHL in humans. In contrast, in an old cell or under disease or ad libitum diet condition, there is a decline in the overall antioxidant defense, which in turn leads to mitochondrial dysfunction and associated hearing loss (Fig. 13.9). In a young cell or under healthy condition, the mitochondrial antioxidant defense systems maintain mitochondrial function through efficiently removing ROS,

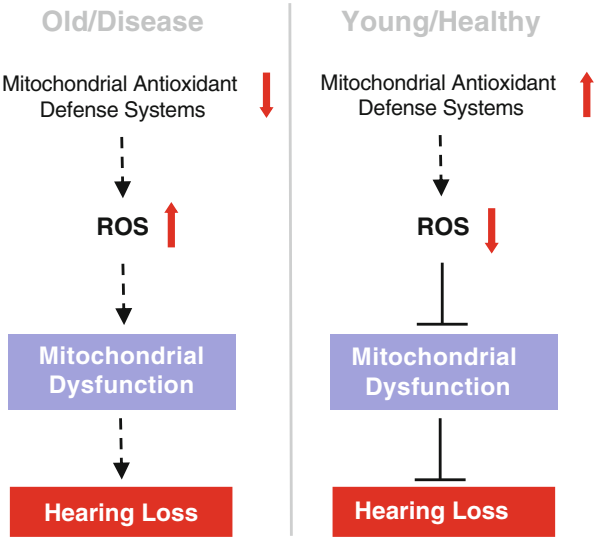

Fig. 13.9 A model for mitochondrial dysfunction in hearing loss. In an old cell or under disease condition (*left panel*), a decline in the mitochondrial antioxidant defense systems results in increased ROS levels. Over time, this in turn leads to mitochondrial dysfunction and drives the progression of hearing loss. In a young cell or under healthy condition (*right panel*), the mitochondrial antioxidant defense systems maintain mitochondrial function through efficiently removing ROS, thereby preventing hearing loss

thereby preventing hearing loss. Given all we have learned in the last two decades, development of drugs that target one of the mitochondrial proteins/enzymes discussed in this chapter to obtain the anti-AHL benefits of dietary restriction seems reasonable.

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Chapter 14
Genetics and Age-Related Hearing Loss

Robert D. Frisina and D. Robert Frisina

Abbreviations

ABR	Auditory brainstem response
ARHL	Age-related hearing loss, presbycusis
AVCN	Anteroventral cochlear nucleus
DCN	Dorsal cochlear nucleus
PCR	Polymerase chain reaction quantitative measure of gene expression
PTS	Permanent threshold shift, permanent hearing loss
PVCN	Posteroventral cochlear nucleus
ROS	Reactive oxygen species, free radicals
SGN	Spiral ganglion neuron, auditory nerve fiber
SNP	Single nucleotide polymorphism

14.1 Introduction

Age-related hearing loss (ARHL)—presbycusis—is the most common communication disorder and neurodegenerative condition of our aging population; and one of the top three chronic medical conditions, along with arthritis and cardiovascular diseases. Mild to moderate ARHL can negatively impact social communication, family relations, and professional productivity, particularly when speech communication is

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required in noisy environments or multi-talker listening situations (Frisina and Frisina 1997). Presbycusis can lead to social/familial isolation and psychological problems such as depression, anxiety, and/or distress. ARHL is often accompanied by tinnitus—ringing of the ears—i.e., perception of phantom sounds in the absence of external acoustic stimuli, which worsens the impact of presbycusis (Rauschecker et al. 2010). There are currently no medical treatments to prevent or decrease the symptoms of ARHL. Therefore, a better understanding of the genetics of ARHL would likely pave the way for novel biomedical therapeutics including development of pharmacological or other forms of interventions. Limited advances have been made concerning the genetics of ARHL for human genes whereas genetic variations in animal models have been fundamental in advancing our knowledge of the neurological consequences of ARHL. Hopefully, increasing information on candidate genes from animal studies will define a path to increase our knowledge of genetics of ARHL in humans. This chapter will summarize current knowledge of genetics of presbycusis in animal models, and emerging evidence on genes related to ARHL in humans.

14.2 Genetics of ARHL in Animal Models

There is a rich history and scientific literature built on animal models, mostly rodents, for understanding normal auditory system function, and what goes awry in cases of ARHL. A wealth of genetic information discovered through use of transgenic and knockout animals have made mice a valuable model in biomedical research. Part of this literature has involved the use of genetically engineered rodent strains, in particular, inbred strains of mice with interesting auditory aging characteristics (Henry 1982; Erway et al. 1993; Johnson et al. 2006). In some cases, as presented below, inbred mouse strains with accelerated ARHL have emerged, which allow for isolating different perceptual and biological characteristics of ARHL; and in a few instances, genes that cause ARHL in mice have been found in certain human populations.

14.2.1 Utilization of Inbred Mouse Strains

Pioneering genetic studies in animal models have yielded valuable insights into the underlying mechanisms responsible for ARHL. For example, one of the most useful mouse strains in this regard has been the C57 (Henry 1982). One of the key components of the electromechanical transduction apparatus of mammalian hair cells, which is a significant part of our mechanism for hearing, are the tip links on the dorsal portions of the stereocilia residing on the top of cochlear inner and outer hair cells (Sotomayor et al. 2010). When sound enters the cochlea, the basilar membrane vibrates, causing a shearing force at the top of the hair cells as they vibrate along

with the surrounding organ of Corti. This sound-induced shearing force opens and closes potassium channels in the stereocilia tip links extremely rapidly, controlling depolarization of the hair cells in response to acoustic stimulation. These hair cell tip links are composed of two proteins, protocadherin-15 and cadherin-23, wherein mutation of the latter's gene causes progressive hearing loss or deafness in certain circumscribed human populations (Astuto et al. 2002; Noben-Trauth et al. 2003; Schultz et al. 2005). The C57 and BALB mouse strains have a mutation in the *CDH23 Ahl* gene, resulting in production of a cadherin-23 protein that is functionally defective, therefore interfering with the flow of potassium ions into the hair cells from scala media. The presence of this defective protein results in an age-accelerated, high-frequency hearing loss in C57, BALB/c, and certain other mouse strains (Johnson et al. 1997, 2000; Willott et al. 1998; Keithley et al. 2004; Zheng et al. 2009), accompanied by significant loss of hair cells (Spongr et al. 1997; Jimenez et al. 1999). For example, the typical C57 mouse has a high-frequency hearing loss of the same magnitude as an elderly human, by the time the mouse is 6 months old. By the time the C57 reaches middle-to-old age, with old age being about 24 months for mice, these mice are profoundly deaf. The DBA mouse strain, which has additional copies of the mutated *CDH23* gene, has an even more rapid ARHL than the C57 strain.

In some sense, a 6-month-old C57 or DBA mouse has an "old" cochlea, but still a "young adult brain," allowing for isolation of certain key aspects of ARHL. For instance, when considering ARHL broadly, changes of hearing with age could be the result of *direct aging of the cochlea*; *direct aging of the parts of the brain used for hearing*—central auditory system; or, they could be linked to age-related plasticity of the central auditory system *due to reduced inputs from the aging cochlea*. This latter phenomenon is sometimes referred to as peripherally induced central effects (Frisina et al. 2001) or peripherally induced brain plasticity. One research strategy has been to compare changes in the central auditory system of aging C57, BALB, and DBA, with other mouse strains, for example, the CBA mouse strain, which loses its hearing more slowly with age like most humans, to tease out direct aging effects from peripherally induced central plasticity with age (e.g., Kazee et al., 1995).

James Willott and colleagues performed many of the pioneering investigations taking full advantage of the rapid peripheral hearing loss of C57s to explore age-induced auditory neuroplasticity. They discovered significant reorganization of the tonotopic maps at key levels of the central auditory system (Willott 1986, 1991; Willott et al. 1988). In the normal mammalian central auditory system, each of the key nuclei, such as the cochlear nucleus, inferior colliculus (auditory midbrain), and auditory cortex, are tonotopically, or cochleotopically organized. This means that high-frequency sound information, from the cochlear base, is represented in one spatial region of each central auditory center; low-frequency information from the cochlear apical turn is represented in a different region; and middle-frequency sounds are processed systematically in a spatial area in between the high and low frequencies. Willott and coworkers discovered that the high-frequency portions of these central auditory maps were rewired to process lower frequency information as the C57 mice aged. Specifically, in the inferior colliculus, ventral areas that normally

process high sound frequencies became increasingly more responsive to lower frequencies as the C57s aged, and their basal cochlea became increasingly dysfunctional. This demonstrates neural plasticity, or axonal sprouting, where the terminal endings of lower frequency neurons migrate into central auditory regions that previously processed higher frequencies.

Consistent with this functional reorganization of the central auditory system in response to reduced high-frequency inputs (declines in high-frequency outputs from the cochlear base), Willott and colleagues observed underlying structural changes (Willott et al. 1985, 1987). Specifically, for aging C57 spiral ganglion neurons (SGNs) and in the anteroventral cochlear nucleus (AVCN), neuron packing density and cell number *decrease* between 1 and 7 months; whereas these values are stable in CBA mice that lose their hearing slowly with age. The AVCN C57 cell declines were most pronounced in the dorsal area of the AVCN where high frequencies are normally coded in the mammalian ventral cochlear nucleus tonotopic map. Consistent with the central plasticity findings of Willott and colleagues, it was also discovered that the efferent feedback system, from the superior olivary complex back to the cochlear hair cells (MOC system), declines much more rapidly with age in C57s compared to CBAs (Frisina et al. 2007).

14.2.2 Excitotoxicity in C57 and CBA Genetic Strains

One of the leading theories of neurodegenerative causation involves neural degeneration due to repetitive use throughout life, sometimes referred to as *excitotoxicity*. This can result in the buildup of reactive oxygen species (ROS), a by-product of energy production from conversion of glucose and oxygen to energy (ATP), ROS, and water in mitochondria. Excitotoxicity can also invoke the undesired buildup of calcium ions (Ca^{++}) inside neurons, since Ca^{++} enters the neuron during synaptic vesicle release. This occurrence is often referred to as *Ca^{++} excitotoxicity* and can be somewhat regulated by changes in concentration of intracellular Ca^{++} regulatory peptides such as calbindin, parvalbumin, and calretinin. Frisina, Zettel, and colleagues have investigated these changes in two of the most useful mouse strains for studies of ARHL, the C57 and CBA. Some noteworthy findings include age-linked declines in calbindin in key nuclei of the central auditory system, such as in the superior olivary complex of C57s and the auditory midbrain of both strains (O'Neill et al. 1997; Zettel et al. 1997). Interestingly, Zettel and coworkers discovered an *activity-dependent upregulation* of calretinin in the CBA inferior colliculus in old CBAs, which was abolished by deafening CBAs as young adults, then allowing them to live to old age (Zettel et al. 2001).

Barbara Canlon's group has also made some of the key discoveries in the area of age-related changes in key calcium-binding proteins in the auditory brainstem, focusing on calbindin, calretinin, and parvalbumin. They utilized a stereological method, the optical fractionator, to determine the total number of neurons and the number of immunostained neurons in the posteroventral and dorsal cochlear nuclei

(PVCN and DCN) (Idrizbegovic et al. 2001, 2003, 2004). Specifically, they found that in aged C57s and BALB mice, there was a significant age-linked decline in the number of neurons, but surprisingly, the number of parvalbumin-stained neurons *increased* with age (C57s only), as did the percentage of neurons showing calbindin (C57s only), calretinin, and parvalbumin staining in the older mice. In addition, correlations were found between peripheral cochlear pathology, e.g., significant loss of hair cells and SGNs with age, and the age changes in the Ca^{++}-binding proteins. In aging CBAs, similar but less dramatic total neuron declines and relative *increases* in parvalbumin and calbindin were found on a much slower time frame in the PVCN and DCN. Interestingly, in the Long Evans rat strain, which lose hearing slowly like CBA mice and most humans, an *upregulation of parvalbumin* was observed in the aging inferior colliculus (Ouda et al. 2008). Taken together, these studies of the aging brainstem auditory system indicate a relative *upregulation* of calcium-binding proteins with age in the cochlear nucleus and inferior colliculus of several strains of mice and rats. These data indicate that calcium-binding proteins act as endogenous protective agents for reducing the aging effects of Ca^{++} excitotoxicity and/or compensate for reductions in cochlear inputs. It could be that the brainstem auditory neurons expressing these Ca^{++} regulatory proteins are *more likely to survive with age*.

Utilizing a copper/zinc superoxide dismutase (Cu/Zn SOD) knockout mouse strain, McFadden and colleagues (1999a, b) shed light on possible roles of antioxidants in slowing down ARHL. Cu/Zn SOD comprises a first-line defense against age-linked free radical (ROS) damage in the cochlea and other tissues. McFadden and coworkers compared auditory sensitivity (ABRs), and hair cell and SGN counts for homozygous, heterozygous, and 129/CD-1 control mice with both copies of the normal *Cu/Zn SOD* gene at 13 months of age (mouse middle age). They found that the homozygotes and heterozygotes showed significant ABR threshold elevations and hair cell and SGN loss, with phenotypic variability the highest in the heterozygotes. They concluded that Cu/Zn SOD deficiencies *increase the vulnerability of the aging cochlea* to damage, most likely through deficits in metabolic pathways that normally scavenge superoxide radicals.

14.2.3 Interactions Between the Genetics of ARHL and Environmental Ototoxicities

Another fascinating use of the different rodent genetic strains has shed light on relations between noise-induced hearing loss and aging. In the field of hearing research, an important question has been: Are aging animals more susceptible to noise exposure than young adults? Ohlemiller and colleagues (2000, 2006) utilized the CBA, C57, and BALB strains to examine this question. Their main findings were that for young adults, BALB mice were most susceptible to noise damage, followed by C57s, then CBAs. These investigators concluded that the effects of the *Ahl* gene mutation that accelerates ARHL may also weaken the cochlea for tolerating a damaging noise exposure. Interestingly, the middle age mice of each of these three

strains were *less vulnerable* to noise damage relative to their younger counterparts of the same strain. On a related topic, Kujawa and Liberman (2009) exposed young adult CBA mice to robust, sustained, sub-noise-damage-threshold levels of loud noise. They then allowed the CBAs to age otherwise normally from 2 months to up to 2 years, tested the hearing of these mice, and sacrificed them for cochlear anatomical studies. They discovered that the noise-exposed mice had normal hearing thresholds, but a greater degree of suprathreshold ABR amplitude suppression, much more hair cell/auditory nerve ribbon synapse cochlear structural damage, and greater loss of SGNs, relative to age- and strain-matched control mice. Their conclusion was that *prolonged*, *subthreshold noise exposures can accelerate certain key aspects of ARHL*, *including SGN neurodegeneration*.

Similar to relations between loud noise exposure and ARHL, interactions for ototoxic antibiotics and presbycusis have been explored. Jochen Schacht and colleagues examined interactions of aminoglycoside ototoxicity in adult CBA, C57Bl, and BALB mice, and Sprague–Dawley rats (Wu et al. 2001). The young adult mice and rats were injected subcutaneously twice/day with kanamycin for 2 weeks. This resulted in ABR threshold shifts of 50–70 dB in the 20–24 kHz range, as well as hair cell loss and vestibular/balance deficits. The strain effects were similar to the noise study presented above, in that the BALB strain was most susceptible, but different in that the CBAs were next, with the C57s showing the smallest of the effects, along with the rats. Lastly, Schacht and coworkers found that administration of the antioxidant 2,3-dihydroxybenzoate could attenuate the kanamycin-induced auditory threshold shifts, and there was a relation with the amount of melanin in the cochlear lateral wall to this antioxidant protection; in this case, more was better.

14.2.3.1 Genetics of Cochlear Age-Related Hearing Loss

In the largest gene expression study of the aging auditory system to date, human or animal model, Frisina, Zhu, D'Souza, and colleagues assessed gene expression changes in the CBA aging cochlea and auditory midbrain. Here, the auditory phenotype (ABR, DPOAE) was measured in 40 CBA mice of different ages, prior to sacrifice. Next, the cochlear duct (organ of Corti, hair cells, lateral wall) and inferior colliculus were dissected and saved for each mouse (the samples from different mice were *not* combined). Then, these 80 samples (40 cochlea, 40 inferior colliculus) were applied to 80 Affymetrix mouse gene chips, which allowed assessment of over 15,000 mouse genes, for each of the 40 subjects of this study. Having gene expression and phenotypic hearing measures on each mouse, allowed for quantitative assessment of the relations between genotype and phenotype. Any genes reported to change with age and hearing loss in the microarrays were verified with quantitative PCR follow-up with portions of the same tissue sample extracts that were utilized for the gene chips. These experimenters examined families of genes that are important in the areas of age-related neurodegeneration and aging sensory systems. Specifically, transcriptional gene expression patterns of 318 apoptosis-related genes were analyzed. Thirty eight probes (35 genes) showed significant age

differences in expression. These gene families included Caspases, B-cell leukemia/lymphoma 2 family, P53, Calpains, Mitogen-activated protein kinase family [MPK], Jun oncogene, Nuclear factor of kappa light chain gene enhancer in B-cells inhibitor-related and tumor necrosis factor-related genes [NFκB] (Tadros et al. 2008). The GeneChip results of 31 genes were validated using the new TaqMan Low Density PCR Array (TLDA). Eight genes showed highly correlated results with the GeneChip microarray data. These genes were: activating transcription factor 3, B-cell leukemia/lymphoma 2, Bcl 2-like 1, caspase 4 apoptosis-related cysteine protease 4, Calpain 2, dual specificity phosphatase 9, tumor necrosis factor receptor superfamily member 12a, and Tumor necrosis factor superfamily member 13b, suggesting they may play critical roles in inner ear aging.

This research group also analyzed changes in immune system genes with age in this overall study. Several strategies for relating these changes to pathway analysis and other innovative biostatistical analyses revealed immune response pathways involved in cochlear ARHL (Xiao et al. 2004; Tra et al. 2011). Specifically, analyses showed that B cell-mediated humoral immune function plays a role in the underlying etiology of presbycusis, similar to certain other neurodegenerative diseases. Other top pathways identified included those involving dendritic antigen-presenting cells, carbohydrate binding, G-protein coupled receptor binding, and epithelial-to-mesenchymal transition pathways.

Donald Caspary and colleagues have previously demonstrated through an elegant series of investigations in the rat auditory brainstem that an age-linked down-regulation of the primary inhibitory neurotransmitter systems takes place, namely GABA and glycine (Ling et al. 2005; Caspary et al. 2008; Wang et al. 2009). Examination of the GABA system in the mouse cochlear gene array study also yielded some new information on this matter (D'Souza et al. 2008). Specifically, expression of GABA-A receptor subunit α6 was *upregulated* with age and hearing loss, whereas subunit α1 declined. Additionally, GABA-A receptor associated protein like-1, and GABA-A receptor associated protein like-2 genes were significantly *downregulated* with age and hearing impairment in the CBA mouse cochlea.

In the antioxidant family the glutathione peroxidase 6, *Gpx6* and Heat shock protein 1, *Hspb1* genes were found to be *upregulated* with ARHL, and the thioredoxin reductase 1, *Txnrd1* gene was *downregulated* in the CBA mouse cochlea (Tadros et al. 2014). These ARHL antioxidant gene expression discoveries open the door for future interventions, where gene expression patterns can be examined and manipulated clinically to reduce pathologies of ARHL at both peripheral and central levels. These biomedical interventions could involve novel pharmacological or gene therapy approaches.

Aquaporins, particularly aquaporin 4 (coded by *Aqp4*), are membrane proteins critical for the movement of water and ion flux across cell membranes, including cells of the inner ear and central auditory system. It was found that in the cochlea *Aqp4* gene expression *declined* with age and hearing loss, and in the inferior colliculus, an initial down-, then upregulation in old age was discovered (Christensen et al. 2009). The authors theorized that these changes in *Aqp4* gene expression represent an age-related disruption of ion flux in the cochlear fluids that are responsible for

ionic gradients important for sound transduction in cochlear hair cells necessary for hearing. In regard to central auditory processing, aquaporin gene expression age changes may affect neurotransmitter cycling involving supporting cells, thus impairing complex sound neural processing with age.

14.2.3.2 Genomics of the Aging Auditory Brain

Serotonin (5-HT) is a monoamine neurotransmitter that modulates neuron discharges in the cochlea, inferior colliculus, and auditory cortex. Specific functions of serotonin exert themselves via specific receptors; and one of those is the serotonin 2B receptor. As another component of the overall CBA gene expression investigation, Tadros and colleagues (2007a), discovered that the serotonin 2B receptor gene was *upregulated* with age in both cochlea and inferior colliculus, and was correlated with functional hearing phenotypic measures. Additional immunohistochemical protein expression studies of inferior colliculus neurons revealed the presence of more serotonin 2B receptors in old CBA mice relative to young adults, particularly in the external nucleus. So, in this case, the gene and protein expressions both increased with age, perhaps to compensate for less serotonin transmitter release in the aging auditory system.

Glutamate is the primary excitatory neurotransmitter in both the peripheral and central auditory systems. Gene expression changes of glutamate and glutamate-related genes may be a key factor in the pathogenesis of ARHL. Tadros and colleagues, as part of the overall CBA mouse microarray investigation, examined age changes for glutamate-related genes in the inferior colliculus (Tadros et al. 2007b). Gene expression of 68 glutamate-related genes was explored using both the microarrays and real-time PCR. Two genes showed consistent differences linked to ARHL: Pyrroline-5-carboxylate synthetase enzyme (*Pycs*) showed *downregulation*, and a high-affinity glutamate transporter (*Slc1a3*) showed *upregulation* with age and hearing loss. Since Pycs helps convert glutamate to proline, its deficiency in old age may lead to both glutamate increases and proline deficiencies in the auditory midbrain, perhaps potentiating glutamate toxicity and a loss of proline neuroprotective effects with age. The upregulation of the *Slc1a3* gene may reflect a cellular compensatory mechanism to protect against age-related glutamate or calcium excitoxicity.

14.2.4 Altering the Time Course of ARHL in Mouse Strains: Interventional Therapeutics

Auditory neuroscientists are starting to test drug or acoustic interventions to alter the course of presbycusis in different mouse strains. Statin drugs have greatly improved the treatment of hypercholesterolemia by inhibiting cholesterol biosynthesis. Their immunomodulatory and anti-inflammatory actions and beneficial

effects on treatment of atherosclerosis are significant. Atorvastatin is one such drug, and Syka and colleagues (2007) administered it to C57Bl/6J mice (10 mg/kg/day in chow diet) for 2 months. The treated mice showed larger amplitudes of distortion product otoacoustic emissions (DPOAE) relative to the non-treated control group, indicating improved survival of outer hair cell function in the cochlea of aging C57Bl/6J mice. They also observed decreased expression of intercellular and vascular adhesion molecules in the aortic wall of the atorvastatin-treated mice, suggesting that the atorvastatin *reduces* endothelial inflammation, thereby improving the inner ear blood supply, and slowing down certain presbycusic mechanisms.

Willott, Turner, and colleagues (Willott and Turner 2000; Willott et al. 2000) pioneered investigations of acoustic modulation of the progression of presbycusis in various mouse strains. Initially, they exposed young adults with accelerated ARHL, including BALB/c mice, to a 70 dB broadband noise for 12 h/day for at least 1 month. They measured ABR audiograms and pre-pulse inhibition of the acoustic startle response to assess the effects of the augmented acoustic environment (AAE) on the time course of ARHL. Relative to the CBA strain, which showed no benefit, the AAE improved ABR audiogram thresholds and PPI responses in the BALB/c and other rapid-ARHL strains, relative to unexposed control mice of the same age and strain. These investigators noted that the beneficial AAE effects did not occur if the onset of the AAE was too late, i.e., after the severe hearing loss had taken place in the BALB/c mice.

Follow-up studies by Turner, Willott, and others revealed substantiating evidence that an AAE can delay key functional and structural aspects of ARHL in the mouse strains with rapid presbycusis, including C57s and DBAs (Willott and Turner 1999; Willott and Bross 2004; Willott 2009; Willott et al. 2010). In addition, structural improvements induced by the AAE were uncovered in DBAs, including preservation of hair cells and AVCN neurons, and *reduction* of AVCN volume declines with age (Willott et al. 2005). Turner and colleagues (2013) also exposed old (22–23 months) CBA mice to AAEs for 6 weeks, and found structural and functional benefits for males, but not females, suggesting influences of hormonal differential effects late in life. Lastly, Michael Kilgard and colleagues have demonstrated that the beneficial effects of AAEs can manifest themselves at the level of auditory cortical neurons, both anatomically and physiologically in Sprague–Dawley rats (Engineer et al. 2004; Bose et al. 2010).

14.3 Human Genetics of ARHL

Perhaps surprisingly, much less is known about human genes involved in ARHL, relative to the animal model research literature. This is fundamentally due to the fact that older people can have a number of environmental factors that impact negatively on their hearing abilities in old age, unlike laboratory rodents that live in a relatively pristine environment, healthwise. These human environmental variables, such as loud noise (see Chap. 7 by Altschuler), health factors such as

cardiovascular disease, diabetes, and smoking (see Chap. 4 by Shirwany and Seidman), occupational exposure to heavy metals (see Chap. 5 by Park), side effects of chemotherapeutic agents (see Chap. 11 by Laurell) or antibiotics (see Chap. 10, by Rybek), nutrition (see Chap. 6 by Spankovich), and aging factors themselves, *can operate independently or in concert with human genetics*, to determine hearing abilities by the time humans reach old age. These environmental hearing loss variables, which are largely unknown or not accurately reported on health-history or hearing health patient/subject questionnaires, greatly complicate the abilities of hearing researchers, human geneticists, and epidemiologists to tease out environmental effects from human genetic etiologies for ARHL. In addition, within the fields of biostatistics, genetics, and epidemiology, there is great debate about the statistical methodologies and bioinformatics tools that are appropriate and valid for separating environmental factors from genetic causation in human studies of aging. Indeed, disagreements about even the number of subjects needed to definitively demonstrate the association between a human gene and an age-linked neurodegenerative disorder like presbycusis is up for vigorous debate, and is quite dependent on experimental design, number of hearing tests involved in the study, and subject selection and screening criteria. So in sum, very little is known about the human genetics of ARHL, with only one gene strongly associated, as explained next.

14.3.1 Pioneering Epidemiological Studies Indicate That ARHL Is Heritable

The Framingham epidemiological study of human aging had some simple hearing measures as part of its experimental design, including pure-tone, audiometric thresholds. Gates and colleagues (1999) analyzed these data in detail and found correlations between the degree of ARHL among family members, such as old parents and their middle age children, as well as among siblings. Interestingly, it was found that a middle age child's degree of ARHL was more related, on the average, to their mother's degree of hearing loss, compared to their father's severity of presbycusis. Since mitochondria are the only cellular organelles that have their own DNA (genes), and we receive all of our mitochondria originally from our mothers' egg, the stronger correlation between mothers and their children for ARHL, indicates involvement of some mitochondrial genes in severity of presbycusis. These Framingham findings have been confirmed in part in other subsequent studies of human hearing and aging (e.g., Karlsson et al. 1997; Christensen et al. 2001; Viljanen et al. 2007), and together they strongly implicate genetic factors in the etiology and progression of presbycusis, with heritability estimates varying between 0.25 and 0.75. With this knowledge in hand, hearing researchers, statisticians, and epidemiologists have begun the quest for identifying human genes that cause, or predispose one to ARHL, which is the topic of the next section of this chapter.

14.3.2 Initial Investigations Are Suggestive but Inconclusive

Huyghe et al. (2008) conducted a cross-sectional family-based study using audiometric data. They utilized principal component analysis, were able to reduce the dimensionality of the multivariate audiometric phenotype and still capture much of the variation, and retained biologically important characteristics of the audiograms. In addition, they carried out a genome-wide association and a linkage scan with high-density single nucleotide polymorphism (SNP) microarrays. No association signals reached genome-wide significance, but linkage analysis yielded a linkage peak at 8q24.13–q24.22, this area of interest was found on chromosome 8, and variation in this region of chromosome 8 was related to audiogram shape in the elderly. Specifically, genetic variations of chromosome 8 were linked to the degree of either: high-frequency hearing loss slope, or degree of concavity of the audiogram shape (the degree of improvement of thresholds at high frequencies). The 8q24.13–q24.22 signal reached genome-wide significance, as assessed by simulations, representing the first locus for an ARHL-related trait. Additional candidate gene association studies have been published since then, as reviewed in Van Eyken et al. (2007), without conclusive identification of a presbycusis gene.

14.3.3 GRM7 *and Human Presbycusis*

Glutamate is the primary excitatory neurotransmitter for synaptic transmission between inner ear hair cells and nerve fibers of the eighth cranial nerve. The *GRM7* gene codes for one of the key proteins of the glutamate receptors on SGNs that synapse with hair cells. Any abnormalities or variations in the normal structure of GRM7 receptor protein could result in distorted information being transmitted to the central auditory system by the SGNs, and hearing impairment, including ARHL. Friedman and An International Group of Colleagues (2009) conducted a 500K-SNP genome-wide association study of a large European cohort of older subjects (Study 1). They identified one haplotype within the *GRM7* gene (OMIM ID: 604101) as harboring a significant risk allele for presbycusis. Friedman and coworkers also reported another, smaller experiment (Study 2), in which the locus was more finely mapped and confirmed in a second European group of subjects. Although this is the first human presbycusis gene to reach genome-wide significance, limitations of their genetic analysis methods using *Z* scores, either a single intronic SNP or the adjacent haplotype block, allowed for some inconclusiveness in their findings.

To seek more definitive conclusions regarding the role of the *GRM7* gene in human presbycusis, Newman, Frisina, Friedman, and their colleagues undertook a further investigation of *GRM7* haplotype block variations in a group of 687 North American subjects over the age of 58 (mean age = 71 years), who underwent the most extensive battery of hearing tests of any study of the genetics of human presbycusis (Newman et al. 2012). This investigative team treated these auditory test

data as quantitative, continuous variables, and employed mixed modeling analyses to explore the relationships of *GRM7* haplotypes and SNP genotypes. They found that *GRM7* alleles are strongly associated primarily with peripheral measures of hearing loss, particularly pure-tone and speech reception thresholds in older adults.

14.4 Summary and Conclusions

Taking advantage of genetic variations in different mouse strains has yielded much valuable information about structural, functional, and genetic mechanisms that change with age in the cochlea and central auditory system. Characteristics of peripherally induced central auditory plasticity have been elucidated utilizing the C57 strain (rapid hearing loss due to cadherin 23 gene mutation) and the CBA strain (slower ARHL), including rewiring and plastic organizational changes in the brainstem auditory system. Modulation of the peripheral and central biomarkers of presbycusis may be possible with both pharmacological and even acoustic biotherapeutic interventions. Gene expression studies in aging CBA mice yield candidate genes involved in presbycusis that can be examined further in human genetics experiments, including families of apoptotic, antioxidant, neurotransmitter, and immune system genes. Much less is known about human genes involved in presbycusis, with only one gene, *GRM7*, which codes for an important glutamate receptor protein involved in hair cell/auditory nerve synaptic transmission, achieving genome-wide significance and confirmatory replication in multiple human subject cohorts. In closing, we hope that the wealth of knowledge about the genetics of presbycusis gleaned from animal models can now impact on human clinical studies and epidemiological investigations, in terms of experimental design and statistical strategies, more directed health questionnaires, more specific audiometric assessments, and of course tighter comparisons with gene assays. Hopefully, the animal findings will now provide more targeted directions for the human work on candidate genes and pathways for age-related hearing loss.

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Chapter 15
Cellular Mechanisms of Age-Related Hearing Loss

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15.1 Introduction: Age-Related Hearing Loss in the Context of Aging

Age-related hearing loss (ARHL), also known as presbycusis, is a progressive, age-dependent deterioration of auditory function reflected in increased response thresholds in conventional auditory tests, particularly in the high-frequency range, along with degraded frequency discrimination and speech comprehension. It is part of the functional decline of aging, although it should not be regarded as an unavoidable consequence of it. ARHL has the potential for being prevented or treated.

ARHL affects approximately 30–40 % of people in their 60s, about 50 % in their early 70s, and more than 65 % from late 70s onwards (Ohlemiller and Frisina 2008; Gopinath et al. 2009; Lin et al. 2011; Yamasoba et al. 2013). This makes ARHL a major health and socioeconomic problem. Although statistical data are fragmentary, ARHL contributes heavily to the burden of hearing impairment, which now affects over 360 million people worldwide (World Health Organization 2014) with a global cost estimated at 2.5–3 % of the US gross national product (GNP) in 2000 (Ruben 2000). Whereas hearing impairment in the first five decades of life profoundly affects educational and job opportunities, productivity, and general life satisfaction, in the aged population it contributes to family and social isolation, depression, and even cognitive decline and dementia (Lin et al. 2013). This growing problem is spurring extensive multidisciplinary research aimed at understanding, controlling, and treating the causes and consequences of ARHL in humans. A major goal is to

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unravel the intricate network of cellular and molecular mechanisms at the origin of ARHL. The expectation is to identify key processes capable of being therapeutically targeted, to prevent, block, or reverse the progress of presbycusis in the broader context of extending healthy aging.

In this chapter we will first address cellular mechanisms that may be involved in the pathogenesis of ARHL in the broader context of systemic aging (for additional review, see Chap. 13 by Someya). This will be followed by a more detailed review of the histopathological and pathogenic basis that provides a mechanistic framework for the understanding of ARHL. We will specifically address the pivotal involvement of mitochondrial dysfunction in ARHL, likely due to the accumulation of mitochondrial DNA (mtDNA) mutations/deletions and the progressive increase of oxidative stress and apoptosis dysregulation, followed by possible therapeutic implications (for additional discussion, see Chap. 16 by Yamasoba). Finally, we will outline the relevance of central auditory changes in ARHL. Rather than providing a comprehensive review, this last section will try to convey the idea of the need of an integrated approach to ARHL that should take into consideration both peripheral and central aging.

15.1.1 Cellular Mechanisms of Aging and Age-Related Hearing Loss

Insofar as ARHL may be viewed as part of the aging process, many factors contributing to general aging (Fig. 15.1) also contribute to a greater or lesser extent to ARHL. In this regard, ARHL may be considered a particular case of accumulation

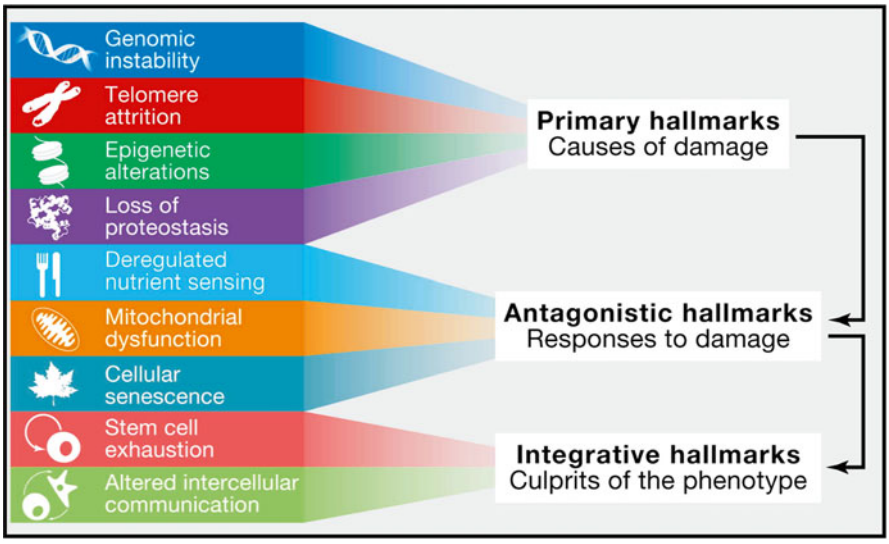

Fig. 15.1 General hallmarks of aging. A detailed explanation of the relevance of this hallmarks and its particularization to ARHL is given in the text (Reproduced with permission of *Cell*)

of cellular damage with time, which is at the origin of the aging process (Kirkwood 2005; López-Otín et al. 2013). In the hearing organ (i.e., the cochlea), this accumulated damage is likely aggravated by the exceptional metabolic requirements (Marcus et al. 1978) needed to maintain the energy-intensive mechanotransduction processes.

It is beyond the scope of this chapter to discuss in detail the multiplicity of factors contributing to aging at the cellular level, their combinations and interactions, which have become known from the use of model organisms from yeasts and worms to primates (for excellent recent reviews, see Gems and Partridge 2013; López-Otín et al. 2013). In Fig. 15.1, an outline of these factors in relation to ARHL is provided in order to place the factors that may contribute to ARHL within the much larger and complex, multifactorial nature of general aging. The relative contributions of each of these factors and potential therapeutic implications for prevention of ARHL have not yet been explored in adequate detail and represent targets for future research.

Factors contributing to aging are interconnected in complex networks. However, identification of hierarchical levels may facilitate operative categorizations. At the top of the proposed "hierarchical network" are the cumulative, combined effects of genome damage and associated genomic instability, altered epigenetic regulation, and defective protein homeostasis (for detailed discussion of the genetics of ARHL, see Chap. 14 by RD Frisina and DR Frisina). An interesting proposal is that these genetic factors may be the main "primers" of the aging process as a whole (López-Otín et al. 2013) (Fig. 15.1).

Accumulation of lesions to the genome, both nuclear and mitochondrial, due to sustained exogenous and endogenous damage and mutations, leading to imbalance of DNA repair and/or maintenance mechanisms, and associated unstable DNA transcription and genomic regulation when these mechanisms fail (Fig. 15.1), is central to age-related cell death (López-Otín et al. 2013). Disruption of nuclear architecture due to mutations in nuclear lamin genes contributes to age-associated genome instability, and mitochondrial DNA damage and mutations are of central relevance in ARHL.

A related primary cause of cellular damage associated with aging is impaired epigenetic regulation (López-Otín et al. 2013) (Fig. 15.1), likely due to damage to the enzymatic systems involved. Altered DNA methylation, aberrant posttranslational modifications of histones, or chromatin remodeling, all may add to genomic instability through faulty transcription, aberrant RNA processing, or defective DNA repair, with profound impact in key cell regulatory pathways (López-Otín et al. 2013). Understanding of epigenetic regulatory mechanisms and their impairment with age in the auditory system is still in its infancy (Provenzano and Domann 2007; Friedman and Avraham 2009; Rossman and Klein 2011), although the possible relevance of alterations in the epigenetic modification of histones during auditory aging has been recently demonstrated with the finding of a switch from acetylated to dimethylated histone H3 in the cochlea of aged mice (Watanabe and Bloch 2013).

A third major, primary factor associated with aging is impaired protein homeostasis (Fig. 15.1) (López-Otín et al. 2013). Sustained endogenous and exogenous cellular stress alters normal protein folding, overriding the molecular chaperones necessary for quality control and repair to assure proper folding and ubiquitination, proteasomal degradation, and regulated autophagy necessary for the disposal of

misfolded proteins. Impaired refolding of damaged proteins or altered degradation mechanisms may lead to accumulation of abnormal proteins and subsequent cellular damage. In this regard, the heat shock transcription factor HSF1, the key regulator of the stress response mediated by heat shock proteins (Hsp) involved in protein folding repair, is intensely expressed in the cochlea (Fairfield et al. 2002). There is evidence that HSF1 and Hsps regulated by this transcription factor, notably those acting as molecular chaperones in the regulation of protein polymerization and folding, like Hsp27, Hsp70, or Hsp90, are involved in the stress response associated with different insults to the cochlea (Altschuler et al. 2002; Sugahara et al. 2003; Fairfield et al. 2004, 2005; Gong et al. 2012). The precise role of these mechanisms in cell death and loss of function (i.e., ARHL) remains elusive however. Pharmacological induction of Hsp expression attenuates age-related progression of hearing loss in some mice strains, albeit in a limited fashion (Mikuriya et al. 2008). Consistent with this, dysregulated autophagic stress with diminished autophagy activity and impaired removal of misfolded, aberrant proteins may also participate in age-related cell damage in spiral ganglion neurons (SGN) (Menardo et al. 2012).

A second set of factors involved in age-related damage likely is limited or exhausted cellular homeostatic protective mechanisms active against cumulative, sustained damage (Fig. 15.1). Such loss of protective mechanisms results in dysregulation of pathways for sensing and adapting metabolism to ongoing cell needs, mitochondrial dysfunction, and altered regulation of cellular senescence (Fig. 15.1) (López-Otín et al. 2013), which are relevant for ARHL as well.

Several signaling pathways involved in the control of cell growth and metabolic and energetic state, aimed at adapting cell needs to changing environmental conditions, show age-dependent dysregulations. Insulin-like growth factor-1 (IGF-1) is a major regulator of cell growth and differentiation, mediating the effects of growth hormone. IGF-1 shares signaling pathways with insulin. Risking oversimplification, insulin/IGF-1 signaling may be seen as an "anabolic sensor" system that couples rates of cell metabolism and growth to nutrient (glucose) abundance. It has been proposed that under conditions favoring damage like those operating during aging, the observed downregulation of insulin/IGF-1 signaling with age may be an adaptation to diminish metabolism and cell growth, thus limiting the cellular consequences of such damage (Schumacher et al. 2008; Garinis et al. 2008; López-Otín et al. 2013) and allowing lifespan extension. Below a given limit, however, such downregulation no longer provides a defensive role in aging but becomes incompatible with cell homeostasis and life. This is an attractive hypothesis that needs further experimental investigation. The impact of IGF-1 signaling in ARHL is complex, but IGF-1 appears to be protective in both mice and humans (for reviews see Bao and Ohlemiller 2010; Varela-Nieto et al. 2013). Studies in IGF-1 knockout mice show that IGF-1 is needed for the maintenance of hearing and that age-related decreasing levels of this growth factor parallel the progression of ARHL (Riquelme et al. 2010).

Another "anabolic sensor" mechanism relevant to aging includes the mTOR (*m*ammalian *t*arget *o*f *r*apamycin) intracellular cascade. mTOR kinases are themselves involved in the cascade of IGF-1 intracellular signaling. However, one of its functional forms, mTORC1, also participates in adapting cell growth and metabolism

to amino acid concentrations through a complex pathway involving the lysosomal membrane (Efeyan et al. 2012). Genetic manipulations or pharmacological inhibition of mTOR with rapamycin extends lifespan in many animal models (Harrison et al. 2009). Increased mTOR activity contributes to age-related obesity (Yang and Ming 2012). Therefore, it seems that intense trophic and anabolic activity signaled through the insulin/IGF-1 or mTORC1 pathways are major contributors to aging, although their specific roles in ARHL remain to be examined in detail.

Two other metabolic state-sensing pathways acting in opposition to the insulin/IGF-1 and mTOR pathways are also relevant to aging. A pathway involving AMPK (5′AMP-dependent protein kinase) signals low-energy availability in cells by detecting high AMP levels. Pathways involving sirtuins (see below) also signal low energy by detecting high NAD^+ levels (Houtkooper et al. 2010, 2012). Therefore, both AMPK and sirtuins detect limited nutrient availability and they signal catabolism predominance in cells. Their upregulation extends lifespan and seems to favor healthy aging in several aging models (López-Otín et al. 2013). Actually, the long known benefits of dietary or caloric restriction (CR) in extending life- and health span in many aging organisms (for a recent review, see Szafranski and Mekhail 2014) seem to be linked to the upregulation of AMPK and sirtuins and downregulation of mTOR and the insulin/IGF-1 pathway, triggered by limitation of nutrient availability, with subsequent positive effects on genomic stability and mitochondrial function. However, negative effects of CR on life and health span also have been reported (Szafranski and Mekhail 2014). This may be related to exhaustion of these metabolic sensors leading to deleterious effects in cell survival with age.

Although a connection between the AMPK pathway and ARHL has not yet been investigated in detail, the role of sirtuins in ARHL has gained particular relevance in recent years. The sirtuins ("*s*ilent mating-*i*nformation *r*egula*t*or*s*," after their role in yeast) belong to a protein family with deacetylase or ADP-ribosyltransferase activities (Houtkooper et al. 2012). As previously mentioned, sirtuin enzymatic activity is linked to the cell energy state through NAD levels so that deacetylation by sirtuins is coupled to NAD hydrolysis. Several of the seven known mammalian sirtuins (Sirt1 to Sirt7) improve aging in mice (Houtkooper et al. 2012). While their actions are complex, many depend on improving genomic stability by epigenetic modifications of histones. It is interesting that a distinct role has been recognized for sirtuins in ARHL (Someya et al. 2010; Han and Someya 2013). CR limits oxidative damage to DNA in the cochlea and slows the progression of ARHL in wild-type mice. However, mice lacking the Sirt3 gene develop ARHL signs even under CR. It seems that Sirt3 directly deacetylates and activates mitochondrial isocitrate dehydrogenase 2 (Idh2), leading to increased NADPH levels and ultimately increased ratio of reduced/oxidized glutathione in mitochondria, thus protecting the auditory receptor from oxidative stress-induced cell death. If the effects of CR in ARHL may be mimicked and more finely controlled by direct (pharmacological) stimulation of sirtuins, this will open the door to new therapeutic targets for ARHL (Han and Someya 2013).

Accumulation of cellular damage caused by the "primary" aging factors previously discussed, along with reduced protective anti-damage mechanisms, can surpass the regulatory homeostatic capabilities of the cell. This leads to the age-damaged

phenotype (Fig. 15.1), when there is also a decline in the regenerative potential of tissues. While mammalian inner ear sensory and neural cells show no regenerative potential, many other tissues of the inner ear do regenerate, and these cells are essential for tissue homeostasis (Menardo et al. 2012), although many details remain to be elucidated.

The previous paragraphs describing the role of genome damage and instability, altered epigenetic regulation, defective protein homeostasis, and reduced protective mechanisms give an idea of the general cellular aging landscape in which ARHL is embedded. Added to these specific mechanisms, there is a complex combination of genetic/intrinsic (e.g., sex, race, particular genes, etc.) and environmental/extrinsic (e.g., history of noise exposure, ototoxic drugs, etc.) factors that also contribute to the development of ARHL (Cruickshanks et al. 1998a, b; Agrawal et al. 2008; Lin et al. 2012; Marlenga et al. 2012; for review, see Yamasoba et al. 2013). In other words, systemic aging is a necessary but not sufficient condition for the development of ARHL. Along with contributions from sex and race, genetic risk factors (Lin et al. 2012; Marlenga et al. 2012) and predisposition to ARHL are reviewed in detail elsewhere (for reviews, see Yamasoba et al. 2013; Chap. 14 by RD Frisina and DR Frisina). It does not come as a surprise that many identified genetic variants and mutations related to susceptibility to ARHL involve genes related to antioxidation (see below) and also to glutamatergic neurotransmission (Sergeyenko et al. 2013; Yamasoba et al. 2013) and excitotoxicity (Pujol et al. 1991; Juiz et al. 1989). As far as environmental factors are concerned, the association of noise exposure and hearing loss is well known (see Chap. 7 by Altschuler and Dolan). In addition to simple additive relationships, noise-induced hearing loss (NIHL) that synergistically accelerates ARHL has more recently been recognized (Kujawa and Liberman 2006). Treatment with ototoxic drugs also results in hearing loss (see Chap. 10 by Rybak and Brenner, and Chap. 11 by Laurell and Pierre). To add further complexity, both noise damage and drug damage to the ear are also subject to genetic susceptibility (see Chap. 8 by Yamashita, Chap. 17 by Kohrman, and Chap. 18 by Green and Raphael). Thus, ARHL is a complex multifactorial disorder in which a lifelong accumulation of insults to the ear on a background of complex genetic predispositions and systemic aging leads to cell damage and hearing impairment (Gates and Mills 2005).

15.1.2 Histopathology of Age-Related Hearing Loss as a Mechanistic Framework

From a histological standpoint, ARHL is characterized by a progressive degeneration of hair cells starting at the basal end or high-frequency coding regions of the cochlea, atrophy and degeneration of the stria vascularis, and a variable degree of degeneration of the primary afferent SGN (Schuknecht and Gacek 1993). Therefore, cell populations in the cochlea involved directly in mechanotransduction (hair cells), in providing the electrochemical driving force for mechanotransduction

(cells in the stria vascularis) or in transmitting and propagating transduced signals from the SGN to the central auditory pathway, may all degenerate and decrease in number and contribute to ARHL (Ohlemiller and Frisina 2008). Whether these universally acknowledged histopathological changes reflect independent, interdependent, or concurrent pathogenic events is an important question with impact on therapeutic strategies. A favored operational concept is that age-associated pathologies of the sensory epithelium, primary sensory neurons, or stria vascularis may arise and evolve independently, although at some point they may merge either by pathophysiological dependency on each other or simply by concurrence. This concept was elaborated by Schuknecht by correlating human patterns of hearing loss with the location of tissue damage and cell pathology in postmortem temporal bones. This seminal work led to the proposal of four major types of human presbycusis, according to the predominant histopathological pattern in relation to changes in auditory thresholds (Schucknecht 1955, 1964; Schuknecht and Gacek 1993; Ohlemiller and Frisina 2008; Schmiedt 2010).

In *sensory presbycusis*, the sensory epithelium is the site of primary damage particularly in high-frequency regions. A variable degree of progressive secondary SGN degeneration follows damage to the sensory lamina (in contrast to primary neural presbycusis). Age-related pathology primarily affecting hair cells may overlap with prolonged accumulation of noise or chemical damage to the auditory receptor making it difficult to separate contributions from preexisting lesions and aging. Therefore, to some extent, there may be a "pathogenic continuum" between accumulation of cell lesions from environmental factors and "true" age-related damage to sensory structures, with important consequences for prevention and treatment strategies.

In *neural presbycusis*, a specific primary loss of SGN with no damage to hair cells was proposed (Schucknecht 1955, 1964; Schuknecht and Gacek 1993; for recent reviews, see Ohlemiller and Frisina 2008; Schmiedt 2010). However, complex trophic relationships between SGNs and their target sensory epithelium challenge the notion of "pure" neural presbycusis. Certainly, there is evidence that SGN may experience progressive degeneration without detectable hair cell loss in young animals, and, conversely, they may survive for extended time periods after hair cell loss (Kujawa and Liberman 2006). Thus, SGN survival or death can be independent of hair cell targets in adulthood. Taken together, there may be a "pure" loss of SGN in neural presbycusis primarily due to aging mechanisms as recently shown in mice (see Boettcher et al. 1995; Schmiedt et al. 1996; Sergeyenko et al. 2013), or it may represent, again, a lifetime accumulation of insults, leading progressively to auditory neuropathy and overlapped with, and indistinguishable of, auditory aging.

In *strial or metabolic presbycusis*, the stria vascularis becomes atrophic and degenerates. The involvement of the stria vascularis in the pathogenesis of ARHL has attracted considerable attention, particularly in recent years. Schuknecht developed the concept that degeneration in the stria vascularis is a major contributor to hearing loss associated with the aging process (Schuknecht and Gacek 1993; Gates and Mills 2005; Schmiedt 2010). Strial pathology seems to be a common feature of ARHL in both humans and animal models. Actually it is the earliest identified

pathological sign in the age-damaged cochlea, first seen in the third decade of life in humans, and it has the highest heritability index (Gates and Mills 2005; Ohlemiller 2009; Fetoni et al. 2011; Yamasoba et al. 2013). However, hearing loss characterized clinically as strial presbycusis sometimes includes loss of hairs cells in the basal turn of the cochlea as the most visible pathology. Some postulate that the hair cell loss could be the result of specific insults such as noise exposure, whereas the strial pathology is purely related to aging (Gates and Mills 2005).

Aged gerbils show a prominent degeneration and atrophy of the stria vascularis (Fetoni et al. 2011). They exhibit a progressive pathology of marginal and intermediate cells and of the Na^+K^+ATPase-positive fibrocytes in the spiral ligament (Spicer and Schulte 2002, 2005; Gates and Mills 2005). This age-dependent degenerative process involves loss of the expression of the Na^+K^+ATPase in the lateral wall and stria vascularis (Schulte and Schmiedt 1992). This ATPase regulates K^+ and Na^+ transport through the lateral wall and, as a consequence, the endocochlear potential (EP) (Spicer and Schulte 2005; Schmiedt 2010; for detailed discussion see Chap. 3 by Ohlemiller). Indeed, aged gerbils exhibit a progressive decline in EP and a disruption of ion homeostasis in the cochlea (Schulte and Schmiedt 1992; Schmiedt 1996, 2010). Thus, in aged animals, degeneration of the stria vascularis can lead to a progressive decline of the EP and impairment of the cochlear amplifier (Gates and Mills 2005; Schmiedt 2010).

Aged-related alterations in the microcirculation of the stria vascularis have been described in several experimental models (Shi 2011). The metabolic rate is high in the stria vascularis (Gates and Mills 2005; Fetoni et al. 2011; Böttger and Schacht 2013). Aged gerbils show atrophy and loss of strial capillaries that gradually expands from the apical to the middle region of the cochlea (Gratton and Schulte 1995). Age-related reduction of cochlear blood flow is exhibited by old gerbils specifically in the lateral wall (Prazma et al. 1990), and the EP decline observed in old gerbils and described above correlates with atrophic capillaries in the stria vascularis (Gratton et al. 1996). Further studies demonstrate age-dependent thickened basal membrane (BM) in 65–85 % with an increase of immunoglobulin and laminin deposition in strial capillaries (Sakaguchi et al. 1997a, b; Thomopoulos et al. 1997), suggesting BM alterations and age-dependent permeability in these vessels. Similarly, aged Fischer 344 rats show decrease in the vascularization of the stria vascularis (Buckiova et al. 2006) accompanied by age-dependent reduction of red blood cell velocity and increased capillary permeability (Seidman et al. 1996). Finally, in 18-month-old C57BL/6 J mice, a decreased expression of the specific endothelial marker VEGF in this tissue is described (Picciotti et al. 2004). These microvascular alterations contribute to the age-related pathology of the stria vascularis and the progressive worsening of ARHL.

Mechanical or conductive presbycusis is characterized by "stiffening" of the basilar membrane, although this remains unproven and under discussion (Gates and Mills 2005; Ohlemiller and Frisina 2008; Schmiedt 2010). However, presbycusis does not present in an isolated form of one of those described above. Instead it comprises a mixture of alterations that likely reflect the summation aging effects in many cell types (Schuknecht and Gacek 1993; Ohlemiller and Frisina 2008). In this regard,

Schuknecht included a frequent form of presbycusis named *mixed* to point out that ARHL is often characterized by a broad cochlear degeneration due to coincident independent causes (Schuknecht and Gacek 1993). Actually, it has been recently proposed (Engle et al. 2013) that in the rhesus monkey, the accumulating number of cochlear pathologies correlates better with audiometric patterns than single pathologies. Finally, Schuknecht established the *indeterminate presbycusis* for those cases where no significant changes were observed in any cochlear structure and its audiogram differed from the conductive one (Schuknecht and Gacek 1993).

It is clear from the above that Schuknecht's histopathological framework is limited and under continuous review. However, it still provides a valid context within which we may view new knowledge of ARHL mechanisms (Ohlemiller and Frisina 2008).

15.1.3 Mitochondrial Mutations and Dysfunction and Its Association with Age-Related Hearing Loss

Mitochondrial dysfunction is central to aging (López-Otín et al. 2013). Actually, altered mitochondrial function is of utmost relevance in ARHL, and it could be at the origin of all the histopathological types of presbycusis described by Schuknecht.

Mitochondria play a central role in the physiology of the cell as the primary source of ATP and are key players in many cellular processes, including calcium signaling and regulation of apoptotic cell death. Mitochondrial metabolism produces free radicals, including reactive oxygen species (ROS) and reactive nitrogen species (RNS) (for detailed discussion, see Chap. 2 by Leeuwenburgh). Both ROS and RNS have signaling roles under normal conditions but are toxic to the cell in excessive concentrations, leading to oxidative stress damage (Fig. 15.2) (Sena and Chandel 2012; Böttger and Schacht 2013). In this regard, mitochondrial dysfunction contributes to a large number of human age-related neurodegenerative diseases such as Alzheimer's and Parkinson's diseases, amyotrophic lateral sclerosis, and Huntington's disease (Lin and Beal 2006) and damage attributed to aging (Lenaz et al. 2006; Lee and Wei 2012; Bratic and Larsson 2013). While there is much evidence supporting the idea that mutations in mitochondrial genomic DNA (mtDNA) and oxidative stress both contribute to aging (Lin and Beal 2006), recent evidence supports a primary role for mtDNA mutations in mammalian aging (Bratic and Larsson 2013; Liochev 2013; Viña et al. 2013). Thus, genetically modified mice undergoing progressive accumulation of mtDNA mutations exhibit an accelerated aging phenotype (Trifunovic et al. 2004; Kujoth et al. 2005). Further research demonstrated that these "mtDNA mutator mice" showed mitochondrial dysfunction unaccompanied by increased ROS production (Trifunovic et al. 2005), suggesting a direct link from the mtDNA mutation to mitochondrial dysfunction and the aging process. We will address the possible association between the mtDNA mutations and ARHL and then the role of ROS generation and oxidative stress in presbycusis.

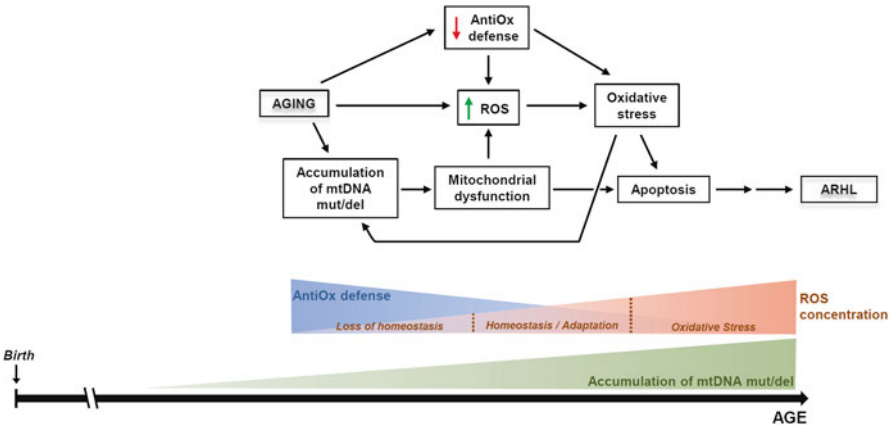

Fig. 15.2 Mitochondrial dysfunction in the pathogenesis of ARHL. Mitochondria have a central role in the pathogenesis of ARHL. During the aging process, many insults damage the cell and are progressively accumulated. In this regard, mitochondrial DNA (mtDNA) mutations and deletions (mut/del) are generated due to the "primary" aging factors and gradually accumulate. This leads to mitochondrial dysfunction that in turn contributes to an oxidative stress situation due to the additive effect of increased toxic levels of ROS and the progressive decline of antioxidant (AntiOx) enzymes that are observed with age in the cochlea. Both processes, mitochondrial dysfunction and oxidative stress, activate apoptosis pathways leading to death of specific cell populations in the auditory receptor contributing to and worsening the aging phenotype. Thus, in the mitochondria of the cochlear cells, there is a progressive accumulation of mtDNA mut/del that significantly contributes to the gradual increase in the ROS levels. Once the toxic level of ROS is reached, oxidative stress ensues, which is also enhanced by the gradual decrease of the antioxidant defense of the cell with age. All these factors will finally activate cell death pathways. The *ROS concentration* sketch is modified from Glasauer and Chandel (2013)

Mitochondrial genomic alterations and ARHL. As stated above, mtDNA deletions and mutations increase with age and also contribute to age-related pathology (Bratic and Larsson 2013; López-Otín et al. 2013). mtDNA mutations are at the origin of a large number of human genetic diseases, many of which include hearing loss (Fischel-Ghodsian 2003; Schon and Przedborski 2011). This suggests a key role for mtDNA mutations specifically in ARHL (Yamasoba et al. 2007). In this regard, it has been estimated that nearly 70 % of patients with mitochondrial genomic disorders also display sensorineural hearing loss (Gold and Rapin 1994; Seidman et al. 2004). In an animal model, 9-month-old "mtDNA mutator mice" showed a significantly greater loss of outer hair cells (OHCs) and SGNs in the basal turn of the cochlea, along with increased ABR threshold shifts, compared to age-matched controls (Kujoth et al. 2005). This suggests that the accumulation of mtDNA mutations in the cochlea as a function of aging may directly lead to hearing impairment attributed to age.

Bai et al. (1997) examined 34 human temporal bone samples, 17 of which belonged to people with ARHL. They found a 4977 base pair (bp) "common aging deletion," which was significantly more frequent in cochlear tissues of ARHL

patients than patients without ARHL. A similar 4834 bp common aging deletion was also found to accumulate with age and to be associated with progressive lower hearing sensitivity in Fischer rats (Seidman et al. 1997). Mutations in the mitochondrial cytochrome oxidase II gene in temporal bones from five ARHL patients have also been described, further supporting the likelihood that sporadic mtDNA mutations also contribute to ARHL (Fischel-Ghodsian et al. 1997). These observations were confirmed in humans by Dai et al. (2004), who found a high incidence of the mtDNA4977 deletion in the temporal bones of ARHL patients (17/34) compared to ears in the age-matched control group (4/19) and those in the young and middle-aged control group (0/14). Perhaps most compelling, a quantitative correlation was established between the level of the mtDNA4977 deletion in human temporal bone samples from ARHL patients and the severity of the hearing loss (Markaryan et al. 2009). These authors also observed a decrease in the expression of the mitochondrial cytochrome c oxidase subunit 3 (COX3) gene in SGNs and an increase in deletions different from the mtDNA common deletion in COX3-deficient SGNs (Markaryan et al. 2010). Taken together, these results are consistent with a causative relationship between the accumulation of mitochondrial genomic alterations with age, mitochondrial dysfunction, and the pathogenesis of ARHL (Fig. 15.2).

15.1.4 Mitochondria, Oxidative Stress, and Age-Related Hearing Loss

Because of the primarily energetic function of mitochondria, ROS are continuously produced in this organelle during the biochemical reactions of the electron transport chain. Superoxide is the primary radical, from which the rest of reactive species are generated through the activity of the cell antioxidant and detoxifying systems (Murphy 2009; Dröse and Brandt 2012). Redox regulatory mechanisms involve several antioxidant enzymes: superoxide dismutases (Cu/Zn-SOD and Mn-SOD), glutathione peroxidase (GPx), glutathione S-transferase (GST), and catalase (Balaban et al. 2005; Kowaltowski et al. 2009; Sena and Chandel 2012). Recent findings demonstrate that ROS also play essential roles in the physiology of the cell by acting in many cell signaling events (Finkel 2012; Ray et al. 2012; Sena and Chandel 2012) related to cellular homeostasis and its adaptation to stress situations (Sena and Chandel 2012). A currently accepted view is that oxidative stress implies an excessive concentration of ROS/RNS due to an imbalance between their generation and the inefficient capacity of the antioxidant systems to scavenge them (Fig. 15.2) (Lee and Wei 2012; Glasauer and Chandel 2013). It has been hypothesized that excessive production accumulates over time, damaging key molecules and cell structures (nuclear and mitochondrial DNA, membranes, and proteins) and resulting in tissue dysfunction during aging (Lee and Wei 2012). Since mitochondria are the major source of ROS (Balaban et al. 2005; Kowaltowski et al. 2009; Brand 2010), the primary effect of oxidative stress we expect would impact on mitochondria, leading to mitochondrial dysfunction with age as seen in several

neurodegenerative diseases and other pathologies (Lin and Beal 2006; Sena and Chandel 2012).

Presbycusis is believed to reflect the progressive accumulation of metabolic alterations due in part to ROS/RNS and the progressive inefficient functioning of mitochondria with age on a background of unusually high metabolic demands in the inner ear tissues (Someya and Prolla 2010; Yamasoba et al. 2013). Although the stria vascularis has the highest aerobic metabolic rate of all cochlear structures (Böttger and Schacht 2013), iOHCs are also vulnerable to oxidative damage because of their high metabolic activity and lower content of antioxidants, like glutathione (Sha et al. 2001).

Recent evidence lends further support to the notion of oxidative stress as a contributing factor in the pathogenesis of ARHL (Someya and Prolla 2010; Yamasoba et al. 2013). There was a significant increase in oxidative stress markers (glutathione-conjugated proteins and 4-hydroxynonenal and 3-nitrotyrosine) and a decrease of the antioxidant scavengers AIF and SOD2 in the cochlea of aged CBA/J mice (Jiang et al. 2007), indicating an imbalance in the redox status of the cochlea with age. On the other hand, *Sod1* deficiency in mice increased the age-related loss of both inner hair cells (IHCs) and OHCs following a base-to-apex gradient, suggesting a correlation between the SOD1 deficiency and the survival of the cochlear sensory cells (McFadden et al. 1999b). Further experiments correlated these hair cell losses with hearing impairment (measured by ABR thresholds) and showed age-related loss of SGN in the 13-month-old homozygous knockout mice (McFadden et al. 1999a). A study with the *Sod1*–null mice showed severe degeneration of SGN at 7–9 months of age, elevated threshold shifts in 12-month-old animals, and thickening of the stria vascularis at 15 months of age, a pattern of results suggesting early ARHL in *Sod1* deficient mice (Keithley et al. 2005). In addition, overexpression of mitochondrially targeted catalase in C57BL/6 J mice delayed the onset of ARHL in mice by delaying hair cell loss (Someya et al. 2009). Finally, a moderate age-related increase in GPx activity has been described in the spiral ligament and stria vascularis in 24-month-old Fisher 344 rats (Coling et al. 2009). Taken together, these results indicate that imbalances in the cellular antioxidant machinery lead to early onset of ARHL and point to oxidative stress as an important contributing factor in its pathogenesis (Fig. 15.2).

If oxidative stress plays a causative role in the pathogenesis of ARHL, it is likely that priming antioxidant mechanisms may reduce cochlear damage and delay onset of pathological changes. However, in contrast to noise-induced and drug-induced hearing loss, where a causal relationship between oxidative stress blocking and improvement in hearing impairment is reasonably well established, in the case of ARHL the link is weaker (Böttger and Schacht 2013). Although many studies have shown effects of antioxidant administration against ARHL, others have not. Seidman (2000) studied the independent effects of several antioxidants (vitamin E, vitamin C, melatonin, and lazaroid) in Fischer 344 rats maintained on lifelong (average, 25 months) supplemented diets. They observed improved auditory thresholds and fewer mtDNA deletions in antioxidant-treated animals, with some agents being more protective than others (see also Chap. 4 by Seidman and Shirwany). Similar

results were obtained with the oral administration of lecithin to 18- to 20-month-old Fischer 344 rats for 6 months (Seidman et al. 2002). Heman-Ackah et al. (2010) fed C57BL/6 mice with a combination of six antioxidant agents (L-cysteine-glutathione mixed disulfide, ribose cysteine, NW-nitro-L-arginine methyl ester, folate, vitamin C, and vitamin B_{12}) and observed a significant decrease in threshold shifts in 12-month-old antioxidant-treated animals. Moreover, in C57BL/6 mice supplemented with the mitochondrial antioxidants α-lipoic acid and coenzyme Q_{10}, a reversal in ARHL was demonstrated, with lower hair cell and SGN cell death (Someya et al. 2009). However, 15-, 18-, or 24-month-old Fischer 344 rats treated with L-carnitine with different methods of administration did not show improvement in progression of ARHL (Bielefeld et al. 2008), and 10-month-old CBA/J mice supplemented with an antioxidant-enriched diet composed of α-lipoic acid, vitamin A, vitamin C, vitamin E, and L-carnitine showed no delay in the onset of presbycusis or loss of hair cells or SGN at 24 months of age (Sha et al. 2012). Additional discussion of therapeutics administered to animal subjects is found in Chap. 16 by Yamasoba.

Taken together, these findings suggest that although oxidative stress is a contributing factor to the pathogenesis of ARHL, it is currently difficult to establish it as a causative factor, without further investigation. In this regard, our lab is studying the otoprotective mechanisms of a combination of antioxidants (vitamin A, vitamin C, and vitamin E) plus magnesium (Mg^{++}). Mg^{++} has been demonstrated to increase inner ear blood flow (Scheibe et al. 2000; Haupt and Scheibe 2002), and its efficacy in attenuating NIHL has been demonstrated (Sendowski 2006). The ACEMg treatment was administered to guinea pigs (Le Prell et al. 2007) and CBA/J mice (Le Prell et al. 2011) showing reduced permanent threshold shifts after NIHL and also reduced threshold shift after gentamicin ototoxicity (Le Prell et al. 2014). Studies in humans indicate that regular dietary intake of antioxidants correlates with lower risks of hearing loss (Durga et al. 2007; Shargorodsky et al. 2010; Choi et al. 2014). Chapter 6 by Spankovich discusses epidemiological data on the role of nutrients in hearing loss prevention in humans.

15.1.5 Apoptosis Regulation and Age-Related Hearing Loss

Several animal models have been used to study the role of apoptosis in the pathogenesis of ARHL (Someya and Prolla 2010; Fetoni et al. 2011). Experiments with old gerbils and mice show that in the aged cochlea, there are various cell types that undergo programmed cell death, such as IHCs and OHCs and supporting cells in the organ of Corti and fibrocytes in the spiral ligament (Usami et al. 1997; Spicer and Schulte 2002). Similarly, aged Fischer 344 rats exhibit increased TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling)-positive cells in both the marginal and basal cell layers of the stria vascularis and cells of the spiral ligament. Apoptosis results in a detachment of both layers. This correlates with altered otoacoustic emissions (Buckiova et al. 2007).

The molecular mechanism involved in such apoptotic death has been extensively studied. Experiments with aging gerbils and rats show downregulation of the anti-apoptotic Bcl family proteins Bcl-2 and Bcl-xL and the activation of pro-apoptotic proteins Bax and Caspase 3 (Alam et al. 2001; Nevado et al. 2006) leading to the hypothesis that the intrinsic pathway of apoptosis may mediate this aged-related cell death. In Fischer 344 rats, Hu et al. (2008) established a temporal sequence of molecular events associated with cell death molecular. Bax expression, release of mitochondrial cytochrome c, and DNA fragmentation take place before the nuclear condensation, followed by activation of caspases 3 and 9, continuing to the degradation of F-actin. On the other hand, gene expression studies in mice demonstrate age-dependent changes of apoptosis-related genes in the cochlea of aging animals (Tadros et al. 2008) and the central role of the mitochondrial pathway of apoptosis via the Bak protein as the key player in this process (Someya et al. 2009). The role of these pathways to cell death is brought together by Sha et al. (2009) who have shown that multiple cell signaling and cell death pathways (both the intrinsic and extrinsic apoptosis and also necrosis) are progressively activated in the cochlea of CBA/J mice with age. Finally, the overexpression of the anti-apoptotic protein XIAP (X-linked inhibitor of apoptosis protein) delays the progression of ARHL and decreases hair cell death in C57BL/6 J mice (Wang et al. 2010).

15.1.6 Cellular Mechanisms of Age-Related Hearing Loss in the Central Auditory Pathways

An understanding of ARHL and possible intervention strategies is incomplete without consideration of cellular and molecular changes associated with auditory aging in the central auditory pathways and their functional consequences. There has been comparatively less attention given to central mechanisms contributing to ARHL, and we strongly believe there is a need for a more integrated view of ARHL, combining peripheral and central mechanisms. Such an approach will not only accelerate understanding of ARHL but will also lead to more efficient management and therapeutic strategies through timely targeting of peripheral and central pathogenic mechanisms (Parham et al. 2011; Gates 2012; Kim and Chung 2013).

Defective processing by central auditory neurons and circuits in ARHL has a dual origin. One is declining peripheral input from the auditory receptor with age. This leads to diminished activity in central auditory neurons which is visible in auditory brainstem response evoked potentials (Fig. 15.3), with consequences for signal propagation and transmission, as well as trophic maintenance of nerve cells. The second is the aging process in the brain itself, which also leads to altered neuronal circuit function, although probably not through identical mechanisms. Both may overlap considerably and even be part of a pathogenic continuum in ARHL. Thus, a major challenge for research on central mechanisms of ARHL is to distinguish between relative contributions and impact of age-declining peripheral input versus direct effects of aging on the central auditory system and to establish

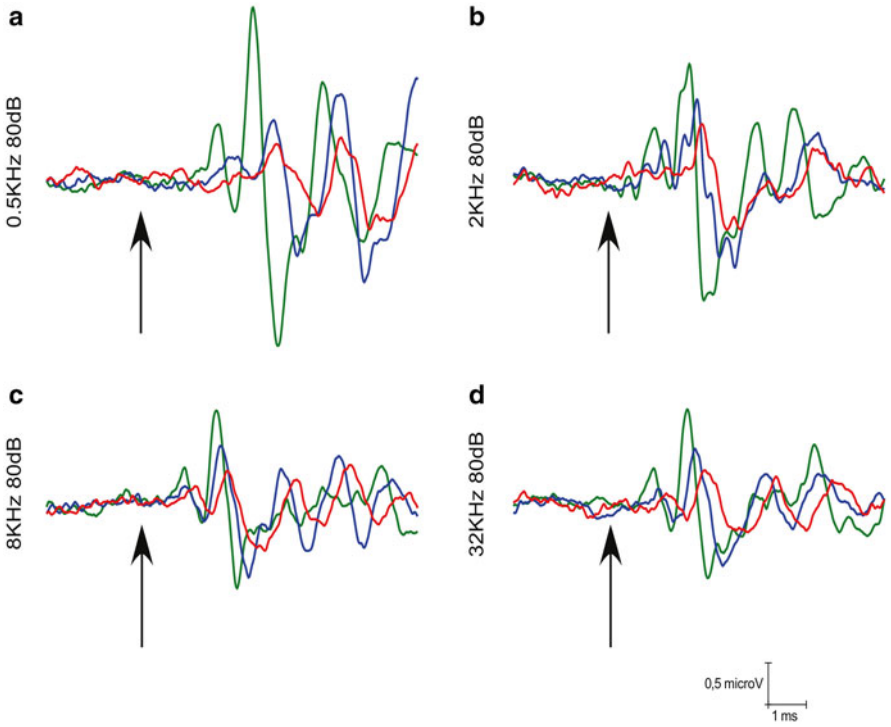

Fig. 15.3 Changes in auditory brainstem responses with age in the rat. An example of ABR recordings in Wistar rats at 6- to 8-month-old (*green*), 12- to 14-month-old (*blue*), and 18- to 20-month-old (*red*). Wave amplitudes decrease at the different frequencies evaluated 0.5 (A), 2 (B), 8 (C), or 32 (D) kHz, and latencies increase as the age of the rats increase. *Arrows* indicate the stimulus onset. Stimulus intensity = 80 dB SPL

their pathogenic sequences and timelines. Sorting the dual origins of the central causes and consequences of ARHL and, equally important, understanding how they interact will have an impact on future therapeutic strategies.

Animal models, in particular rodent strains with various onset, severity, and patterns of hearing loss, have been very useful to dissect out the contributions to central ARHL mechanisms of age-diminished peripheral input versus central aging (see Ohlemiller and Frisina 2008 for review and their chapters in this book). There is evidence that the progression of age-related loss of synaptic input from the auditory receptor affects neuronal survival, trophic support, and signal propagation and transmission (Fig. 15.3), with greatest severity of structural changes in caudal nuclei of the auditory brainstem. Neurons in the cochlear nucleus (CN) are primary recipients of synaptic endings from auditory nerve axons, and therefore the first to "sense" diminished peripheral input. There is an overall reduction of neuron number, packing density, and reduced cell size, distributed tonotopically and consistent with the time course and progression rate of the peripheral hearing loss (Willott et al. 1987, 1992).

Early loss of high-frequency hearing in the C57 mouse strain (Willott et al. 1987) leads to early and stable structural changes in the CN. Delayed hearing loss, as in the CBA mouse (Willott et al. 1987), corresponds with delayed structural changes in aged CN neurons of similar nature. It is interesting that these peripherally driven effects on neuronal structure do not affect uniformly all CN neuronal populations, with octopus cells in the posteroventral CN being particularly resilient to limited auditory nerve input (Willott and Bross 1990). The type of neuron and its pattern of synaptic inputs seem to determine neuronal survival and trophic maintenance in the presence of limited peripheral input with age. However, it is also interesting to note that mouse strains (Willott et al. 1987) or those humans having a significantly larger number of neurons in the CN (Hinojosa and Nelson 2011) seem to be more prone to neuronal loss attributable to presbycusis, suggesting an added genetic component to peripherally driven neuronal loss. In the auditory pathway rostral to the CN, neuronal loss attributable to auditory aging is much more limited or not present (Shim et al. 2012; Willott et al. 1994). Neuronal body size diminishes or remains stable "upstream" of the CN, at least in the inferior colliculus (IC) (Willott et al. 1994; Kazee et al. 1995). Therefore, it seems that trophic support or survival of many CN neuron types is severely compromised by age-dependent reduction in direct peripheral inputs carried by the auditory nerve. However, at more rostral levels of the auditory pathway, beyond the primary inputs, diminished peripheral activity has much more limited impact on net cell survival, supporting the view that trophic signals differ at different levels of the auditory pathway, with possible consequences for the aging process.

As a consequence of damage to neuronal cell bodies, particularly in the CN, axonal degeneration is expected. However, the extent and rate of age-related degeneration of axons traveling in auditory tracts is unclear. Axonal connections from the CN to the inferior colliculus (IC) are not significantly reduced in mice with early signs of ARHL at any age (C57, Willott et al. 1985). However, in mice with mild ARHL, connections from the CN to the IC are significantly reduced with age (CBA; Frisina and Walton 2001, 2006). The number of fibers in the human lateral lemniscus is also reduced at advanced ages (Ferraro and Minckler 1977, cited in Ohlemiller and Frisina 2008). Regardless the extent and rate of axonal degeneration, connections from the CN to more rostral auditory nuclei carry degraded signals (reviewed in Frisina and Walton 2006) as a consequence of aging (Fig. 15.3). Among other things, as a consequence of high-frequency loss, there are rearrangements in the tonotopic map of more rostral nuclei, like the IC (Willott et al. 1991), with high-frequency regions becoming more sensitive to lower-frequency sounds. The nature of this peripherally induced "rewiring" is unknown, but it could add to limited frequency discrimination. In the auditory cortex, plastic reorganizations of the tonotopic map also take place after age-related high-frequency hearing loss, with low frequencies expanding its representation to higher-frequency regions (Willott et al. 1993). The reorganization in connectivity underlying such age-related auditory cortex plasticity is not known. Such plastic reorganizations in the auditory midbrain and forebrain do not seem to take place in rodent models aging with good hearing preservation (Willott et al. 1993).

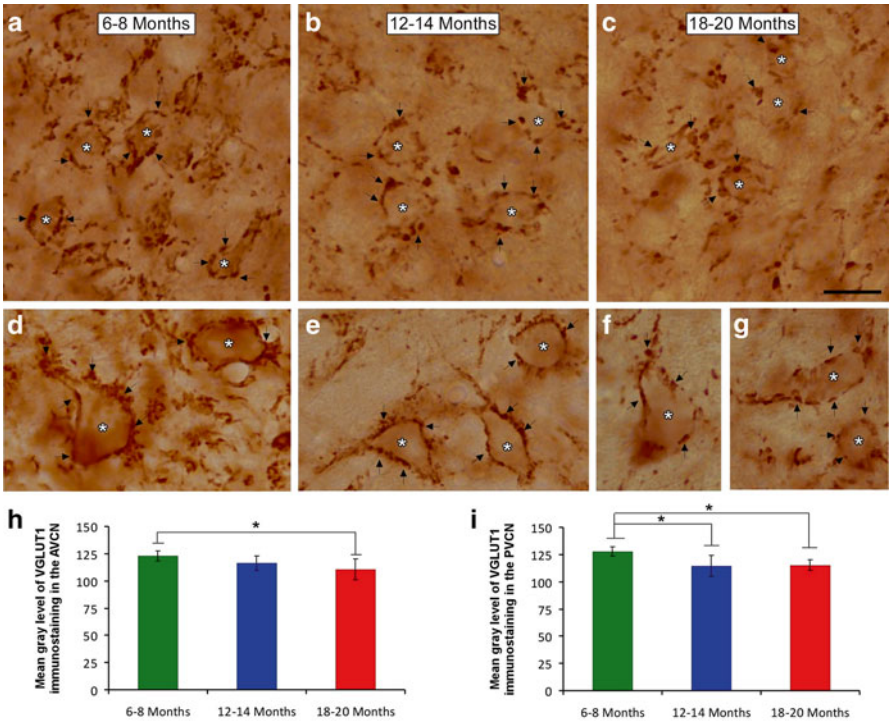

Fig. 15.4 Changes in central excitatory neurotransmission with age in the rat. Vesicular glutamate transporter-1 (VGLUT1) immunoreactivity in the AVCN (A–C) and PVCN (D–G) in 6- to 8-month-old (A, D), 12- to 14-month-old (B, E) and 18- to 20-month-old (C, F and G) Wistar rats. Immunopositive profiles (*arrows*) in 6- to 8-month-old rats (A, D) seem more abundant than in 12- to 14-month-old (B, E) and 18- to 20-month-old (C, F and G) rats. Bar graphs indicate the mean gray level of VGLUT1 immunostaining in the AVCN (H) and PVCN (I). The mean gray level in the 18- to 20-month-old rats is lower compared to the 6- to 8-month-old rats. Asterisks in A–G indicate neurons. Scale bar represents 25 μm in C $*p < 0.05$

Directly related to altered neuronal trophic support and structural and functional connectivity are age-related modifications or adaptations of synaptic circuits, reflecting both peripheral and intrinsic central changes, and relevant to understanding and management of central mechanisms of ARHL (Frisina and Walton 2006; Caspary et al. 2008) (Figs. 15.4 and 15.5). Rearrangements of synaptic structure and size take place in the CN with aging (Keithley and Croskrey 1990; Helfert et al. 2003). However, there seems to be no net synaptic loss (Helfert et al. 2003), which might be a consequence of aberrant reactive synaptogenesis (Keithley and Croskrey 1990), due to loss of primary inputs from the aging cochlea. In the IC, however, loss of excitatory and inhibitory endings predominates (Helfert et al. 1999; Kazee et al. 1995). Considering that synaptic loss and synaptic functional abnormalities are hallmarks of brain aging (Shankar 2010; Bano et al. 2011), it is difficult to distinguish those reflecting age-related peripheral changes and intrinsic central changes.

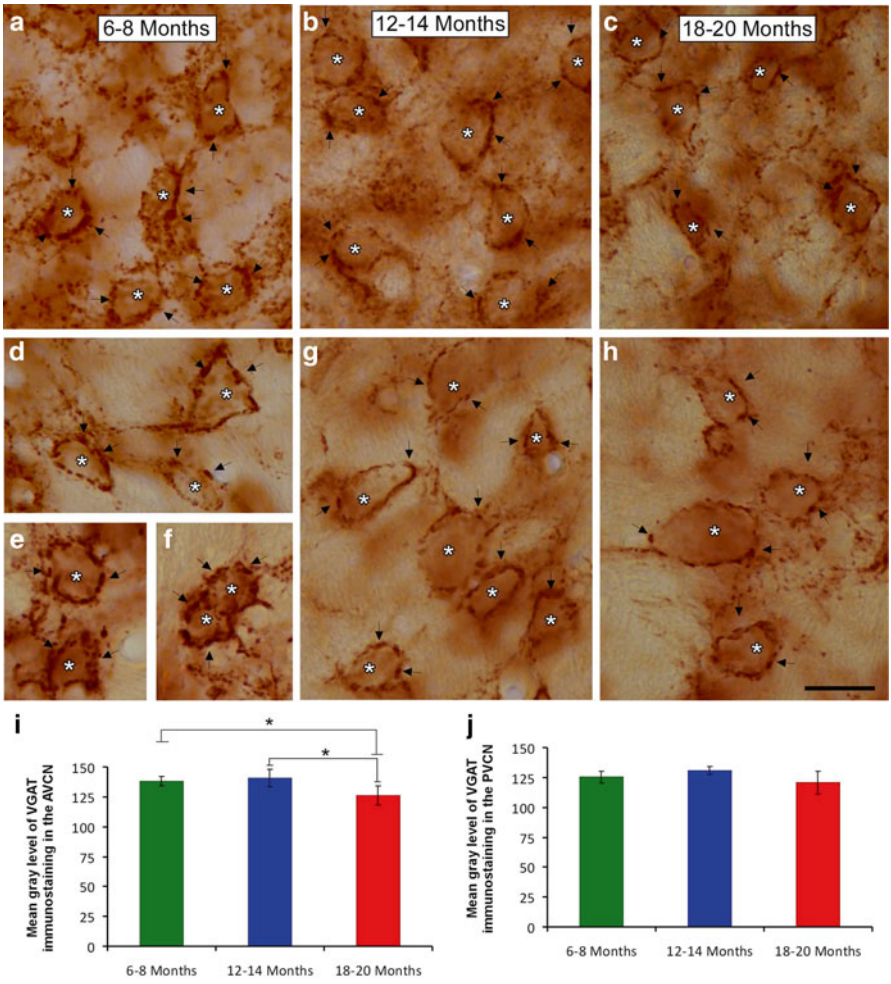

Fig. 15.5 Changes in central inhibitory neurotransmission with age in the rat. Vesicular GABA transporter (VGAT) immunostaining in the AVCN (A–C) and PVCN (D–H) in 6- to 8-month-old (A, D–F), 12- to 14-month-old (B, G), and 18- to 20-month-old (C, H) rats. An apparent decrease in the immunostaining of large and small inhibitory synaptic endings is observed with age, with VGAT profiles (*arrows*) distributed more abundantly in the 6- to 8-month-old rats (A, D–F) than in both the 12- to 14-month-old (B, G) and 18- to 20-month-old (C, H) rats. The mean gray level in the AVCN (I) and PVCN (J) in 18- to 20-month-old rats is significantly lower compared to both the 6- to 8-month-old rats and the 12- to 14-month-old rats. Asterisks in A–H indicate neurons. Scale bar represents 25 μm in H. $*p < 0.05$

It has been reported that the structure and number of synapses is well preserved in the IC of the CBA mouse, which shows well-preserved hearing until late in life (Kazee and West 1999). This is in contrast with the C57 mouse and other rodent strains with earlier and/or more severe hearing loss in which synaptic loss in the IC

is greater (Kazee et al. 1995; Helfert et al. 1999). This indicates that age-dependent auditory peripheral deficits influence central synaptic survival, although the contribution of brain aging itself remains to be fully explored (Caspary et al. 2008).

Age-driven structural rearrangements in the distribution and/or number of central auditory synapses may be connected with adaptive synaptic changes (Figs. 15.4 and 15.5), to restore a lost delicate balance between excitation and inhibition (reviewed in Caspary et al. 2008). In the CN, excitatory input from the auditory nerve is reduced during aging as a result of degeneration of SGN (reviewed in Bao and Ohlemiller 2010) or altered synaptic transmission with deficient synaptic glutamate handling (Fig. 15.4) (Alvarado et al. 2014), lower glutamate release, and changes in glutamate receptor composition and kinetics (Wang and Manis 2005). To maintain homeostasis inhibition is downregulated to maintain original levels of activity (Caspary et al. 2008; Alvarado et al. 2014). Glycine-mediated inhibition is particularly affected in the CN (reviewed in Caspary et al. 2008). Age-related decreases in glycine (Willott et al. 1997), inhibitory amino acid synaptic vesicle transporters (Fig. 15.5) (Alvarado et al. 2014), and synaptic release of glycine (Xie and Manis 2013) are mirrored by changes in postsynaptic GlyR subunit expression and composition and altered glycine binding to GlyR (Willott et al. 1997; Caspary et al. 2008; Wang et al. 2009). Briefly, at the synaptic level, glycinergic inhibition decreases with age (Xie and Manis 2013). As a result, discharge rate of CN neurons increases, and dynamic range and ability to code signals that change very fast in time is degraded (Caspary et al. 2005, 2008). Although gamma-aminobutyric acid (GABA) is relevant to CN neuronal inhibition and signal processing, GABAergic neurotransmission in the CN is more limited. Actually, GABAergic markers do not change significantly with age in the CN (Raza et al. 1994; Sharma et al. 2014). Such a selective downregulation of glycinergic neurotransmission in the CN during ARHL is interesting since GABA and glycine typically co-localize and co-release from inhibitory synaptic endings in the CN (Juiz et al. 1996; Lim et al. 2000). In addition to altered inhibitory neurotransmission, changes in intrinsic properties reported in some neuronal classes (bushy cells, Wang and Manis 2006) may also contribute to degraded excitability in the CN during aging. These observation are consistent with the view that the hallmarks of ARHL, i.e., reduced speech discrimination in noisy backgrounds (Anderson et al. 2012), begin with age-altered synaptic circuit properties at the very first central auditory relay stations of the auditory pathway.

Excitatory-inhibitory synaptic imbalances with diminished inhibitory neurotransmission appear to be a constant feature of ARHL along the auditory pathway. In the superior olivary complex (SOC) which receives input from both CN and is involved in sound localization, inhibition also is impaired (Finlayson 1995; reviewed in Caspary et al. 2008). Reductions in GAD activity, the rate-limiting enzyme involved in GABA synthesis, have been reported in the nuclei of the lateral lemniscus (NLL) of aged Fischer 344 rats (Raza et al. 1994) although functional implications are unknown.

Age-related excitatory-inhibitory imbalances are especially well documented in the IC (Caspary et al. 2008; Ohlemiller and Frisina 2008). Virtually all available

evidence points to a severe age-dependent decrease in inhibitory GABAergic function in the IC as a pivotal central mechanism of ARHL (Caspary et al. 2008), although interspecies variations may exist (Gleich et al. 2014). GABA and/or GAD levels measured in neurochemical assays or by immunocytochemistry show large age-related decreases in the IC (reviewed in Caspary et al. 2008 and Ohlemiller and Frisina 2008). Brain aging may contribute significantly to the decline of presynaptic markers of GABAergic function, but the share of peripheral and central contributions to diminished IC GABAergic inhibition is unknown (Caspary et al. 2008). GABA receptors also change their composition and binding properties with age in the IC, which may underlie compensatory mechanisms (Caspary et al. 2008). Gene and protein expression of subunits assembling ionotropic GABAA receptors responsible for "fast" inhibition have been reported to be downregulated to a greater or lesser extent in the IC with aging (Gutiérrez et al. 1994), with the notable exception of the gamma1 and alpha3 subunits which are upregulated (Milbrandt et al. 1997; Caspary et al. 1999). GABAA receptors enriched in gamma1 subunits may allow larger Cl influx, which may be part of a mechanism of compensation for impaired GABA release (Caspary et al. 2008) and as well as reduced receptor density (Gutiérrez et al. 1994). Changes with age in the binding properties of the picrotoxin site of the GABAA receptor also indicate modulation of GABAA responses with aging in the IC (Caspary et al. 2008). GABAB receptors, responsible for "slow" modulatory responses, have also been found to be diminished in the aging IC (Milbrandt et al. 1994). Taken together, this evidence supports a massive net downregulation of GABAergic inhibition in the IC with aging. Significant decreases in inhibitory responses of single IC units within their receptive fields, increases in the width of the response above threshold, and reduced precision in the temporal processing of AM and FM sounds (Caspary et al. 2008) may originate at least in part in defectively compensatory GABAergic downregulation. This could lead to degradation of temporal, spectral, and binaural processing in the IC with aging.

Evidence of excitatory-inhibitory imbalances, suggestive of maladaptive homeostatic plasticity during auditory aging, has also been found recently in the auditory thalamus of aged Fischer Brown Norway rats. An age-related reduction in GAD67 was found in the medial geniculate body (MGB) indicating reduced GABA synthesis. High-affinity GABAA receptors containing the delta subunit were reduced almost to half, and the membrane distribution of the high-affinity alpha4/delta GABAA receptor subunit combination was altered with age. This high-affinity GABAA receptor mediates long-lasting tonic inhibition which was found to be reduced with aging in the MGB, along with fast, phasic GABAergic inhibition (Richardson et al. 2013). Age-diminished tonic inhibition in the auditory thalamus may alter excitability, increasing temporal uncertainty and reducing coding fidelity.

Aging in the auditory cortex also involves maladaptive homeostatic synaptic plasticity, with inhibition imbalance, primarily involving GABAergic neurotransmission (Caspary et al. 2008). GAD is significantly decreased in the primary auditory cortex, actually more so than in other regions of the neocortex (Burianova et al. 2009; Ling et al. 2005). This likely involves downregulation of GABA synthesis rather than actual loss of GABAergic neurons in the cortex. Whether this represents

an effect of aging, peripheral hearing, loss or both is unclear, although there seem to be no differences between aged rats from strains differing in progression of hearing loss (Burianova et al. 2009), indicating brain aging itself. Defective GABA synthesis may impact synaptic release, and this is mirrored by age-altered expression of GABAA receptors in neurons in the auditory cortex. The expression of protein subunits involved in the assembly of functional GABAA receptors in the cortex (alpha1, beta2, gamma2) has been recently found to be downregulated as a consequence of aging, with concomitant upregulation of the alpha3 subunit, suggesting compensatory changes in receptor composition and kinetics (Caspary et al. 2013), comparable in part to those found in the IC where the expression of the alpha3 subunit is also increased with age. However, compensatory GABA inhibition may include circuit-specific mechanisms, as increases in gamma1 subunit expression found in the IC (Caspary et al. 2008) do not seem to be present in the auditory cortex.

GABA downregulation with inadequate postsynaptic balancing results in profound age-altered inhibition at the level of the auditory cortex. This is likely reflected in age-related threshold increase, increases in spontaneous and evoked activity in the auditory cortex (Juarez-Salinas et al. 2010), modified receptive fields (Turner et al. 2005; Juarez-Salinas et al. 2010), and loss of spectral precision and directional selectivity. This decreases the ability to localize sounds and extract salient signals from complex acoustic backgrounds, resulting in degraded speech understanding (Caspary et al. 2008). There seems to be a direct association between age-related disruption in GABAergic neurotransmission and changes in signal coding at the level of the auditory cortex.

The critical role of Ca^{++} in the regulation of multiple basic cellular processes, including neuronal excitability, is well known, and Ca^{++} dysregulation is a central element of cellular aging (López-Otín et al. 2013). Neuronal Ca^{++} regulation is likely altered at all levels of the aging central auditory pathway. Calcium-binding proteins (CaBP) are essential to maintain intracellular Ca^{++} concentration within the narrow margins required for physiological functions. Major CaBP in the brain are parvalbumin, calbindin (calbindin D28k), and calretinin. There have been numerous reports of changes in the expression of these three CaBP in the aging auditory pathway (Burianova et al. 2009; see Ouda et al. 2008 and Ouda and Syka 2012 for review). A particularly relevant example is provided by parvalbumin, the most abundant CaBP in "core" areas of the auditory pathway, involved in fast auditory sensory processing. With few exceptions, parvalbumin expression increases with age in many neurons throughout the auditory pathway. This is probably a consequence of neuronal aging, aggravated and/or induced by diminished peripheral auditory inputs (Idrizbegovic et al. 2003; Ouda et al. 2008; Gray et al. 2014). Large increases in parvalbumin levels have been recently reported in the rhesus monkey during aging from the cochlear nucleus to the auditory thalamus (Gray et al. 2013, 2014; Engle et al. 2014). This highlights even more the relevance of altered neuronal Ca^{++} homeostasis in human ARHL. Increased levels of parvalbumin in neurons and/or increased numbers of neurons expressing parvalbumin during aging, along with concomitant changes in the expression of other CaBP, may be essential for buffering excessive intracellular free Ca^{++}. Excessive neuronal

accumulation of intracellular Ca^{++} during aging may originate from mechanisms such as mitochondrial dysfunction (see above) or unbalanced excitability with increased Ca^{++} influx (Cataldi 2013). Unusually high expression of parvalbumin in aging auditory neurons, actually higher than in other aging sensory systems, along with changes in other CaBP, may protect neurons from toxic excess of intracellular free Ca^{++}. On the other hand, a very large number of GABAergic neurons, particularly in the IC and auditory cortex, also express parvalbumin. Increased parvalbumin expression in GABAergic neurons might alter their excitability through Ca^{++} buffering, thus contributing to inhibitory-excitatory imbalances during aging. However, regulatory compensation of Ca^{++} homeostasis in the aging auditory pathway may have other levels of complexity. The auditory cortex is unique in that changes in parvalbumin levels are not homogeneous, but rather field- and cortical-layer specific (Martin del Campo et al. 2012). Although parvalbumin expression is increased in deep cortical layers, it tends to diminish in GABAergic interneurons (Ouda and Syka 2012; Ouellet and de Villers-Sidani 2014), with no apparent neuronal loss. The varying age-related changes in auditory cortical inhibitory circuits may reflect a need-based plasticity. Thus, parvalbumin levels in GABAergic interneurons in the auditory cortex of the rat recover normal levels with training (de Villers-Sidani et al. 2010).

Besides adaptive changes in the expression of CaBP, other mechanisms also may contribute to Ca^{++} regulation with aging. It is interesting that primary excitatory synapses on spherical cells in the CN with age adaptively switch the normal subunit composition of their postsynaptic receptors from highly permeable to Ca^{++} to receptors impermeable to Ca^{++} (AMPA class of glutamate receptors enriched in the GluR2 subunit; see Wang and Manis 2005). This could be a very interesting general adaptive synaptic mechanism of aging which remains to be fully explored.

Central mechanisms of auditory aging arise from both defective synaptic input reflecting an aging auditory receptor and the effects of aging in the brain. Although both may overlap considerably, establishing the sequence and hierarchies of both mechanisms at different levels of the auditory pathway will impact future treatments of presbycusis. Central presbycusis may be partially seen as a synaptopathy with altered neuronal excitability that could be subject to pharmacological treatments. Behaviors and agents, e.g., antioxidants, that may prevent stress-induced peripheral pathology through life may provide the best strategy to protect the inner ear from age-related changes.

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Chapter 16
Interventions to Prevent Age-Related Hearing Loss

Tatsuya Yamasoba

Abbreviations

ABR	Auditory brainstem response
ALCAR	Acetyl-L-carnitine
ARHL	Age-related hearing loss
CR	Caloric restriction
DPOAE	Distortion product otoacoustic emission
ED	Every day feeding
EOD	Every other day feeding (Monday, Wednesday, and Friday)
GNL	Gluconolactonase
HC	Hair cell
HEI	Healthy eating index
HFD	High-fat diet
KO	Knockout
MLR	Middle-latency response
mtDNA	Mitochondrial DNA
NIA	National Institute on Aging
PTA	Pure-tone average
ROS	Reactive oxygen species
SD	Standard deviation
SGC	Spiral ganglion cell
SMP	Senescence marker protein
SOD1	Cu/Zn superoxide dismutase

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SOD2	Manganese superoxide dismutase
SV	Stria vascularis
TEOAE	Transient-evoked otoacoustic emission
UW	University of Wisconsin at Madison
VC	Vitamin C
WT	Wild-type

16.1 Introduction

Age-related hearing loss (ARHL), also known as presbycusis, is a universal feature of mammalian aging and the primary pathology includes the hair cells (HCs), stria vascularis (SV), and spiral ganglion cells (SGCs) in the cochlea as well as the central auditory pathways (for detailed discussion of mechanisms of age-related hearing loss, see Chap. 15 by Juiz et al.). ARHL in humans is characterized by a decline of auditory function, such as increased hearing thresholds, poor speech perception especially under noise condition, and poor temporal processing (Gates and Mills 2005; Humes et al. 2012). An increase in pure-tone hearing threshold appears at higher frequencies, then extends toward lower frequencies, and is accelerated with age. The onset, extent, and rate of hearing loss vary significantly among elderly subjects because of the differences of their genetic and environmental backgrounds. Epidemiological studies have demonstrated four categories of risk factors of ARHL in humans, including cochlear aging, environmental factors such as noise exposure, genetic predisposition, and health comorbidities such as cigarette smoking and atherosclerosis (Yamasoba et al. 2013). Genetic investigation has identified several putative associating genes, including those related to antioxidant defense and atherosclerosis (Uchida et al. 2011) (for detailed discussion of genetics of age-related hearing loss, see Chap. 14 by R.D. Frisina and D.R. Frisina).

A number of animal models of ARHL have been developed to allow detailed study of disease progression and causes. Recent studies using such animal models have effectively provided insight in cellular and molecular mechanisms that contribute to ARHL (for detailed discussion of biochemical pathways and molecular targets, see Chap. 13 by Someya). In brief, a growing body of evidence strongly suggests that cumulative effect of oxidative stress can induce damage to macromolecules such as mitochondrial DNA (mtDNA) and that the resulting accumulation of mtDNA mutations/deletions and decline of mitochondrial function play an important role in inducing apoptosis of the cochlear cells (see Yamasoba et al. 2013). Calcium signaling, stress responses, sex-specific hormones, and glucocorticoid signaling pathways may also contribute to the development of ARHL (Kidd Iii and Bao 2012). Various interventions according to these hypotheses have been performed to prevent or delay ARHL mostly in animals. This chapter describes main findings in reported studies of interventions in animals and humans and discusses the optimal strategy to prevent or delay ARHL.

16.2 Interventions Assessed in Animals

16.2.1 Antioxidants

It has been suggested that oxidative stress could be increased with age in the cochlea. For example, glutathione peroxidase activity was reported to increase in the SV and spiral ligament in the cochlea of aged Fisher 344 rats (Coling et al. 2009). In the organ of Corti of CBA mice, glutathione-conjugated proteins, markers of H_2O_2-mediated oxidation, began to increase at 12 months of age and 4-hydroxynonenal and 3-nitrotyrosine, products of hydroxyl radical and peroxynitrite action, respectively, were elevated by 18 months, whereas antioxidant proteins (mitochondrial apoptosis-inducing factor) and enzyme (manganese superoxide dismutase: SOD2) decreased by 18 months (Jiang et al. 2007).

Animal models susceptible to oxidative stress have been shown to display an acceleration of aging-related phenotypes in the cochlea, suggesting that the cochlea is very sensitive to reactive oxygen species (ROS)-induced damage. For example, mice missing the gene encoding Cu/Zn superoxide dismutase (SOD1) show acceleration of ARHL (McFadden et al. 1999; Keithley et al. 2005). Compared with wild-type (WT) mice, SOD 1 homozygous and heterozygous knockout (KO) mice exhibited significant elevations of auditory brainstem response (ABR) thresholds and greater HC loss at 13 months (McFadden et al. 1999). In addition, reduced thickness of the SV and severe degeneration of SGCs were observed in middle-aged SOD1 knockout mice (Keithley et al. 2005), although overexpression of SOD1 did not protect against ARHL except at 24 months of age (Keithley et al. 2005). SOD2 heterozygous KO mice did not show such acceleration of ARHL, although expression of 8-hydroxydeoxyguanosine was significantly increased in the SGC and SV in heterozygous SOD2 KO mice compared to WT mice (Kinoshita et al. 2013). Unlike humans and guinea pigs, rats and mice can synthesize vitamin C (VC). Senescence marker protein 30 (SMP30)/gluconolactonase (GNL) KO mice cannot synthesize VC, but can metabolize exogenously administered VC. The SMP30/GNL KO mice given water containing only 37.5 mg/L VC showed significant reduction in VC level in the inner ear and exhibited increased ABR thresholds and a decreased number of SGCs at 10 months compared to WT mice. Such changes did not occur when SMP30/GML KO mice were given water containing sufficient amount (1.5 g/L) of VC (Kashio et al. 2009). Conversely, overexpression of catalase in the mitochondria reduced oxidative DNA damage in the cochlea and slowed ARHL in C57BL/6 mice (Someya et al. 2009). These findings suggest that oxidative damage in the cochlea reflects an age-related decline in the antioxidant defenses and/or an age-related increase in ROS levels plays a crucial role in the development of ARHL.

Based on the responsiveness of the cochlea to ROS, several groups have examined if ARHL could be prevented or delayed by adding exogenous antioxidants. The results are significantly varied among reports; some studies showed clear benefit provided by antioxidant treatment (Seidman 2000; Seidman et al. 2002; Le and Keithley 2007; Someya et al. 2009; Heman-Ackah et al. 2010), but others showed no effect (Bielefeld et al. 2008; Sha et al. 2012).

In studies using rats, (Seidman 2000) conducted a randomized prospective study over a 3-year period, in which 2-month-old Fischer 344 rats were given vitamin E, VC, melatonin, or lazaroid for approximately 2 years and observed that the antioxidant-treated animals had better auditory sensitivities and a trend for fewer mtDNA deletions compared with placebo subjects. Seidman et al. (2002) also examined the effects of lecithin, a polyunsaturated phosphatidylcholine that plays a rate-limiting role in the activation of numerous membrane-located enzymes including SOD and glutathione, on ARHL. When Harlan-Fischer rats aged 18–20 months were divided into controls and experimental group supplemented orally for 6 months with lecithin, lecithin-treated animals showed significantly better hearing sensitivities, higher mitochondrial membrane potentials, and fewer common aging mtDNA deletion in the cochlear tissues including the SV and auditory nerve compared to controls.

In studies using mice, several antioxidants have been reported to be effective to ameliorate ARHL. Someya et al. (2009) fed C57BL/6 mice with control-diet or diet containing one of 17 antioxidant compounds [acetyl-L-carnitine (ALCAR), α-lipoic acid, carotene, carnosine, coenzyme Q10, curcumin, tocopherol, EGCG, gallic acid, lutein, lycopene, melatonin, proanthocyanidin, quercetin, resveratrol, or tannic acid] from 4 to 15 months of age and observed that ARHL measured at 15 months of age was nearly completely prevented by α-lipoic acid and coenzyme Q10 and partially by *N*-acetyl-L-cysteine, but not by other compounds. Heman-Ackah et al. (2010) assigned C57BL/6 mice to early (3–12 months of age) or late (6–12 months of age) treatment group or control group and treatment groups of mice were fed with a combination of six antioxidant agents that target four sites within the oxidative pathway including L-cysteine-glutathione-mixed disulfide, ribose-cysteine, NW-nitro-L-arginine methyl ester, vitamin B12, folate, and ascorbic acid. Both the early and late treatment groups exhibited significantly smaller ABR threshold shifts compared to control group at 12 months of age.

Antioxidants also have been reported to slow ARHL in dogs. Le and Keithley (2007) fed aged beagle dogs (10–15 years old) with a high antioxidant diet for the last 3 years of their life. The diet contained DL-α-tocopherol acetate, L-carnitine, DL-α-lipoic acid, ascorbic acid, and 1 % inclusions of each of the followings: spinach flakes, tomato pomace, grape pomace, carrot granules, and citrus pulp. The dogs fed with this high-antioxidant diet showed less degeneration of the SGCs and SV compared to dog fed a control-diet.

In contrast to these positive findings, several researchers failed to show the effectiveness of antioxidant treatment against ARHL. For example, (Bielefeld et al. 2008) treated F344/NHsd rats at the age of 15 or 18 months with ALCAR by oral gavage once daily for 90 days and those at the age of 24 months with ALCAR by intraperitoneal injection daily for 1 month, but ABR thresholds of the three sets of ALCAR-treated animals were never significantly different from their matched controls before, during, or after the treatments. Sha et al. (2012) fed CBA/J mice with antioxidant-enriched diet containing vitamins A, C, and E, ALCAR, and α-lipoic acid from 10 through 24 months of age and observed significantly increased antioxidant capacity of the inner ear tissues, but not amelioration of ARHL or loss of the HCs and SGCs.

These findings indicate that supplementation with certain antioxidants can prevent/slow ARHL in animals, but that antioxidant therapy is not always effective. The different outcomes may be accounted for by differences in the type and dosage of antioxidant compounds, timing, and duration of the treatment, species, and strains.

16.2.2 Caloric Restriction

Caloric restriction (CR) has been shown to extend the lifespan of most mammalian species and to slow the rate of aging in mammals. In laboratory rodents, CR can delay the onset of age-related diseases, such as prostate cancer, nephropathy, cataracts, diabetes, hypertension, and hyperlipidemia (Sohal and Weindruch 1996; Mair and Dillin 2008). It is difficult to determine whether CR has beneficial effects on longevity and age-related diseases in humans because there are no validated biomarkers that can serve as surrogate markers of aging and because it is impractical to conduct randomized, diet-controlled, long-term survival studies in humans. Nonetheless, data from epidemiologic studies suggest that CR may have beneficial effects on the factors involved in the pathogenesis of primary and secondary aging and life expectancy in humans (see Yamasoba et al. 2013).

Two different dietary feeding regimens of CR have been widely used because of their reproducible abilities to retard the rates of aging in rodents (Mattson et al. 2003). In an "every day feeding (ED)" regimen, the animals receive food daily, but are limited to a specified amount, which is usually 30–50 % less than the ad libitum consumption by the control group. In an "every other day feeding (EOD)" regimen, the animals are deprived of food for a full day, every other day, and are fed ad libitum on the intervening days. As for housing regimens, the use of individual housing for all animals in CR studies has been widely used since it allows for the food intake of each animal to be controlled with great accuracy (Pugh et al. 1999).

The preventive effect of CR against ARHL has been inconsistent across reports (see a review by Someya et al. 2010a). In a study of 18-month-old AU/Ss mice (Henry 1986), CR animals were fed standard lab chow ad libitum EOD (only on Monday, Wednesday, and Friday), while control mice were fed ad libitum daily for a 17-month period. CR increased the AU/Ss life span by 44 % and the CR mice weighted an average of 14 % less than the control mice. At 18 months of age, both control and CR mice displayed significant threshold elevations at 2 through 64 kHz, but the mean thresholds of CR mice were significantly lower than those of controls at all the frequencies measured. In AKR strain, however, the same dietary feeding and housing regimens starting from 2 months of age until 4 months of age did not show differences in hearing thresholds between control and CR animals. CR did not increase the life span of the AKR mice, but the CR mice weighed less than the control (22.8 ± 1.1 g in CR vs. 29.2 ± 1.2 g in control) (Henry 1986). This may be due to the fact that the onset of ARHL in AKR strain occurs at a very young age (1–2 months of age), which may be too early for CR to be beneficial. CR also delayed the onset of ARHL in the AU, CBA, and B6 strains of mice, but not in the DBA, WB,

or BALB strains (Willott et al. 1995). Someya et al. (2007) observed that C57B/6 mice that received CR by 15 months of age retained normal hearing and showed no obvious cochlear degeneration and a significant reduction in the number of TUNEL-positive cells and cleaved caspase-3-positive cells in the SGCs compared to age-matched controls; microarray analysis also revealed that CR downregulated the expression of 24 apoptotic genes, including *Bak* (BCL2-antagonist/killer 1) and *Bim* (BCL2-like 11), suggesting that CR could prevent apoptosis of the cochlear cells. Interestingly, high-fat diet (HFD) given for 12 months, which is opposite to CR, elevated hearing thresholds at high frequencies and increased ROS generation, expressions of NADPH oxidase and uncoupling protein, accumulation of mtDNA common deletion, and cleaved caspase-3 and TUNEL-positive cells in the inner ear of Sprague–Dawley rats. After a 12-month diet, the body weights were significantly greater for the rats in the HFD (857.9 ± 27.4 g) than for the rats fed a basic diet (664.6 ± 13.9 g) and the levels of plasma triglyceride, total cholesterol, nonesterified fatty acids, and H_2O_2 were significantly higher in the HFD group than in the control group (Du et al. 2012).

Beneficial effects by CR have also been reported in rats. Fischer rats that were calorie restricted to 70 % of the control intake beginning at 1 month of age and then housed for 24–25 months showed significantly better ABR thresholds, reduced HC loss, and decreased mtDNA common deletion in the auditory nerve and SV of the cochlea compared to non-calorie-restricted, matched controls (Seidman 2000). Mannström et al. (2013) reported that 73 % of female Sprague–Dawley rats on a 70 % CR had preserved Preyer reflexes and only modest degeneration of the SV at 30 months of age, whereas in age-matched, ad libitum fed animals only 15 % had detectable Preyer reflexes and showed a marked thinning, cellular degeneration, and loss of cell processes in the SV.

The beneficial effect of CR against ARHL has been equivocal in rhesus monkeys. There have been two large studies ongoing at the University of Wisconsin-Madison (UW) and the National Institute on Aging (NIA) designed to test if CR can retard the rates of aging and delays the onset of ARHL. In the UW study (Fowler et al. 2002), 33 rhesus monkeys were on CR diets and 35 controls were fed ad libitum daily; CR was maintained at 30 % less than the calories consumed by the ad libitum group. At the time of the auditory tests, the ages of the monkeys were between 11 and 23 years and the monkeys had been in the dietary study 3–9 years. ABR and middle-latency response (MLR) analyses were used to monitor the progression of AHL, and the peak IV amplitude of ABR waves of CR female monkeys was significantly larger compared to control females. In addition, the binaural peak IV amplitude of ABR waves decreased significantly faster with age for control monkeys compared to CRs. The mean ABR threshold by click stimuli in CR male monkeys was lower than that in control males, but the difference was not statistically significant. These results suggest that some components of auditory function in the aged monkeys could be maintained by CR.

In the NIA study (Torre et al. 2004), 26 rhesus monkeys were on CR diets and 24 controls were fed ad libitum to investigate the effects of CR on the onset of ARHL. CR was maintained at 30 % less the calories consumed by the ad libitum group.

At the time of the tests, the mean ages of the CR and control monkeys were 20.4 and 18.7 years respectively, and monkeys had been in the CR study 12–13 years at the time of testing. Dietary feeding and housing regimens similar to those of the UW study were employed to study CR in monkeys at the NIA. ABR, MLR, and distortion product otoacoustic emission (DPOAE) analyses were used to monitor the progression of ARHL. No significant effects of CR, however, were found on any parameters examined. At the time of the hearing tests, the age range of the control monkeys was 13–32 years, while that of the CR monkeys was 13–36 years. Therefore, the lack of effect of CR might be due to the fact that some of the monkeys were still relatively young at the time of the tests and had not reached the age (25 years) at which ARHL begins to be clearly evident with hearing tests, and where CR is most likely to have an effect on auditory function.

An alternative possibility is related to the ability to detect differences from the control groups, which differ across the UW and NIA studies. Control monkeys at UW are fed ad libitum whereas control monkeys at NIA are maintained on a mildly restricted diet to avoid obesity. There is currently an ongoing debate as to the extent to which this difference in control animal diet has influenced the ability to detect differences in longevity, given that differences are detected only in the UW data, with no difference in the NIA subjects. In other words, because the control animals in the NIA study already had some CR imposed, it may have been difficult to detect any additional accrued benefit with more significant CR in the NIA animals during the auditory testing by (Torre et al. 2004). This is clearly an important issue, and additional work in rodent models may help to clarify these key questions.

The underlying mechanisms for the CR-associated benefits are still under debate. CR failed to reduce oxidative DNA damage and prevent ARHL in C57B/6 mice lacking the mitochondrial deacetylase Sirt3, a member of the sirtuin family (Someya et al. 2010b). In response to CR, Sirt3 directly deacetylated and activated mitochondrial isocitrate dehydrogenase 2 (Idh2), leading to increased NADPH levels and an increased ratio of reduced-to-oxidized glutathione in mitochondria. In cultured cells, overexpression of Sirt3 and/or Idh2 increased NADPH levels and protected from oxidative stress-induced cell death. These findings strongly suggest that at least a primary mechanism underlying the beneficial effects of CR is mediated by ROS-antioxidant systems and that Sirt3 is essential in enhancing the mitochondrial glutathione antioxidant defense system in the cochlea during CR.

16.2.3 Other Treatments

Calcium homeostasis has been suggested to contribute to ARHL since elderly women using calcium channel blockers were reported to have better hearing in a cross-sectional analysis of 357 subjects including 184 females (Mills et al. 1999). HCs and SGCs have several types of calcium channels, including L- and T-type voltage-gated calcium channels. Lei et al. (2011) recently reported that ARHL was delayed and the SGCs were preserved in C57BL/6 mice missing the gene encoding

the $Ca_v3.2$ T-type calcium channel and that wild-type B6 mice treated with anticonvulsant drugs from a family of T-type calcium channel blockers had significant preservation of hearing thresholds and SGNs compared to untreated controls.

Stress response proteins are known to play a role in maintaining cochlear function. Mikuriya et al. (2008) demonstrated that expression of stress-responsive proteins HSP70 and HSP110 was greater in the cochleae of CBA/N compared to DBA/2J mice, which are prone to ARHL, and that addition of a heat shock protein inducer, geranylgeranylacetone, to the diet ameliorated ARHL in DBA/2J mice, although the protection was specific to the apical portion of the cochlea.

Statin, which is widely used to treat hypercholesterolemia due to their ability to inhibit cholesterol biosynthesis, also has immunomodulatory and anti-inflammatory effects and positive effects on the treatment of atherosclerosis and its complications. Syka et al. (2007) demonstrated that C57BL/6J mice treated with atorvastatin (10 mg/kg per day in chow diet) for 2 months showed larger amplitudes of DPOAEs compared to non-treated controls.

Other potential interventions may target glucocorticoid signaling pathways, sex-specific hormone pathways, and glutamate-signaling pathways (Kidd Iii and Bao 2012). The glucocorticoid signaling pathway may contribute to ARHL since deletion of β_2-subunit of the nicotinic acetylcholine receptor has been reported to result in accelerated ARHL associated with SGC degeneration (Bao et al. 2005). Sex-specific hormone pathways are also considered important because sex-specific differences have widely been observed in ARHL in humans and animals and because a combination hormone replacement therapy using estrogen and progestin increased the incidence of ARHL (Price et al. 2009). Other interventions have also been proposed, including augmentation of acoustic environment, electrical stimulation to restore the endocochlear potential, and salicylate therapy (Bielefeld et al. 2010).

16.3 Epidemiological Data from Humans

16.3.1 Antioxidants

The role of nutrition in healthy hearing was reviewed in detail in Chap. 6 by Spankovich; here we focus more specifically on the interaction between diet and ARHL. Gopinath et al. (2011a) examined if dietary and supplement intakes of antioxidants could influence development of ARHL. They analyzed dietary data collected in the Blue Mountains Hearing Study, a population-based survey of ARHL; specifically, they calculated intakes of α-carotene, β-carotene, β-cryptoxanthin, lutein, zeaxanthin, lycopene, vitamins A, C, and E, iron, and zinc as assessed using a semiquantitative food-frequency questionnaire. They measured hearing in 2,956 participants, aged 50 years or older, examined during 1997–2002, and defined ARHL as the pure-tone average (PTA) thresholds at frequencies including 0.5, 1.0, 2.0, and 4.0 kHz > 25 dB HL. After adjusting for age, sex, smoking, education, occupational noise exposure, family history of hearing loss, history of diagnosed

diabetes, and stroke, they found that each standard deviation (SD) increase in dietary vitamin E intake was associated with a 14 % reduced likelihood of prevalent hearing loss and that subjects in the highest quintile of dietary vitamin A intake had a 47 % reduced risk of having moderate or greater hearing loss (>40 dB HL) compared to those in the lowest quintile of intake. However, dietary antioxidant intake was not associated with the 5-year incidence of hearing loss.

Spankovich et al. (2011) examined the data from an overlapping subset (2,111 adults aged >49 years) of those who participated in the Blue Mountains Hearing Study during the years 1997–1999 and whose data were included in the analysis of (Gopinath et al. 2011a). Spankovich et al. (2011) measured univariate and multivariate associations and observed that higher carbohydrate, vitamin C, vitamin E, riboflavin, magnesium, and lycopene intakes were all significantly associated with larger (better) transient-evoked otoacoustic emission (TEOAE) amplitude and better PTA thresholds whereas higher cholesterol, fat, and retinol intakes were significantly associated with lower (poorer) TEOAE amplitude and worse PTA thresholds.

Choi et al. (2014) analyzed cross-sectional PTA threshold data from 2,592 participants aged 20–69 years from National Health and Nutrition Examination Survey (NHANES) 2001–2004 in the USA and examined associations between intake of antioxidant vitamins (daily beta-carotene and vitamins C and E) and magnesium and hearing thresholds. When examined individually, modeled as quartiles, and after adjustment for potential confounders, higher intakes of beta-carotene, vitamin C, and magnesium were associated with better PTA thresholds at both speech and higher frequencies, and higher intakes of beta-carotene or vitamin C combined with higher magnesium compared with lower intakes of both nutrients were significantly associated with better PTA thresholds at higher frequencies.

It should be noted, however, that several reports have shown no relationship between diet and hearing or no relationship between specific nutrients and hearing. For example, (Shargorodsky et al. 2010) prospectively evaluated the association between intake from foods and supplements of vitamins C, E, beta-carotene, B12, and folate and the incidence of hearing loss using the subjects who participated in the Health Professionals Follow-Up Study enrolling 51,529 male dentists, optometrists, osteopaths, pharmacists, podiatrists, and veterinarians aged 40–75 years at baseline in 1986. The participants filled out a detailed questionnaire about diet, medical history, and medication use. These questionnaires have been administered every other year, and the 20-year follow-up exceeds 90 %. The 2004 long-form questionnaire included a question regarding whether the participant had been professionally diagnosed with hearing loss, and if so, the date of diagnosis. Those who reported hearing loss diagnosed before 1986 or cancer and those who reached age 75 during follow-up were excluded. As a result, 3,559 cases of hearing loss were identified, and there was no significant association between vitamin intake and risk of hearing loss, although total folate intake was associated with a reduced risk of hearing loss among men 60 years and older.

Because review of the literature reveals mixed support regarding specific nutrients and the relationship between single nutrient intake and hearing (see Spankovich and Le Prell 2013), one should be very careful regarding how to interpret findings in single nutrient analyses. If a diet is healthy with respect to one vitamin, it is likely

healthy with respect to other vitamins. When the analysis is limited to a small number of nutrients, there may be important confounding covariates, which could be missed if single nutrient analysis is used. This has led to the use of the Healthy Eating Index (HEI) which provides an overall dietary quality estimate. When the relationship between HEI and hearing level was analyzed in 2,366 participants who participated in the National Health and Nutrition Examination Survey 1999–2002, there was a statistically significant negative relationship between HEI and PTA thresholds for high frequencies (Spankovich and Le Prell 2013). Specifically, those that had diets that more closely met US Dietary Guidelines for Americans had better hearing at higher frequencies, with no reliable relationship at lower frequencies associated with speech perception (Spankovich and Le Prell 2013). At the higher frequencies, the overall effect was small; the most robust effects—which were approximately 5-dB, were observed in the oldest subject of participants, suggesting the potential for an effect of healthy eating to increase across the lifespan. Given the cross-sectional nature of those data, prospective studies would have significant utility in identifying and/or confirming any causal relationships.

Several studies have examined the effects of antioxidants on ARHL in humans, but the number of subjects enrolled has been small and the results conflict across studies. When 60 patients between 65 and 76 years old, with ARHL, received oxidized coenzyme Q10 (160 mg/day), vitamin E (50 mg/day), or placebo for 30 days ($n=20$ each), those who received coenzyme Q10, but not those who received vitamin E, showed a significant improvement of hearing thresholds at 1,000, 2,000, 4,000, and 8,000 Hz compared to the controls (Guastini et al. 2011). When 46 patients ranging in age from 70 to 91 years were given rebamipide (300 mg/day), vitamin C (600 mg/day), and α-lipoic acid (60 mg/day) daily for at least 8 weeks, hearing levels after treatment were significantly improved at all frequencies (Takumida and Anniko 2009). In addition, 34 (37.0 %) ears showed a clinically significant improvement of hearing (≥10 dB) at 125, 250, 500, 1,000, 2,000, 4,000, and 8,000 Hz (Takumida and Anniko 2009). In contrast, when 120 patients aged 60 years or older and with ARHL received one of four treatments for 6 months [ginkgo biloba (120 mg/day), α-lipoic acid (60 mg/day) plus vitamin C (600 mg/day), papaverine chlorhydrate (100 mg/day) plus vitamin E (400 mg/day), or placebo (starch pills)], no statistically significant change in the hearing thresholds was observed after treatment with any of the tested drugs (Polanski and Cruz 2013). Because ARHL is slowly progressive and the HCs and SGCs do not regenerate, it is unclear why and how hearing thresholds were improved by administration of certain antioxidants in the studies by (Guastini et al. 2011) and (Takumida and Anniko 2009).

16.3.2 Statins

Olzowy et al. (2007) examined if atorvastatin, 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitor, could slow down the progression of ARHL in 50 patients (60–75 years old) with ARHL and moderately elevated serum cholesterol,

who were randomly assigned to treatment with either atorvastatin (40 mg/day orally) or placebo in a double-blind manner. Hearing thresholds after 7 and 13 months showed no significant differences between groups, although tinnitus score continuously improved in the atorvastatin group while it slightly deteriorated in the placebo group.

In contrast, (Gopinath et al. 2011b) reported a beneficial effect of statin against ARHL. They assessed associations between dietary intake of fats and certain food groups (butter, margarine, and nuts) with the prevalence, incidence, and progression of ARHL and also investigated the link between statins and hearing loss in a population-based survey of ARHL, the Blue Mountains Hearing Study comprising 2,956 participants aged 50 years or older. After multivariable adjustment, the likelihood of prevalent hearing loss increased from the lowest to the highest quartile of dietary cholesterol intake and among persons self-reporting statin use, a 48 % reduced odds of prevalent hearing loss was observed.

16.3.3 Omega-3 Polyunsaturated Fatty Acid

Gopinath et al. (2010) also examined if dietary intake of omega-3 polyunsaturated fatty acid (PUFA) could prevent or slow the development of ARHL. They collected dietary data in the Blue Mountains Hearing Study comprising 2,956 participants aged 50 years or older and calculated PUFA and fish intakes by using a semiquantitative food-frequency questionnaire. There was an inverse association between total omega-3 PUFA intake and prevalent hearing loss and between long-chain $n-3$ PUFAs and incident hearing loss. Participants who had ≥2 servings of fish/week compared with participants who had <1 serving of fish/week had a significantly reduced risk (42 %) of developing ARHL at follow-up. There was an association between consumption of ≥1 to <2 servings/week of fish and a reduced risk of a progression of hearing loss. These findings suggest that dietary intervention with omega-3 PUFAs could prevent or delay the development of ARHL.

16.4 Discussions

As discussed above and in other chapters, it is now strongly suggested that oxidative stress and mtDNA mutations/deletions play a crucial role in the development of ARHL. Two interventions that have been extensively investigated in animals are (1) raising them with a calorie-restricted diet and (2) enhancing their antioxidant defenses with exogenous antioxidants. It is now evident that supplementation with certain antioxidants can prevent/slow ARHL in animals, but antioxidant therapy is not always effective. This is also true in animals treated with CR. The reasons for such different outcomes are unknown, but differences may be explained by the type and dosage of antioxidant compounds, the extent and regimen of CR, the timing and

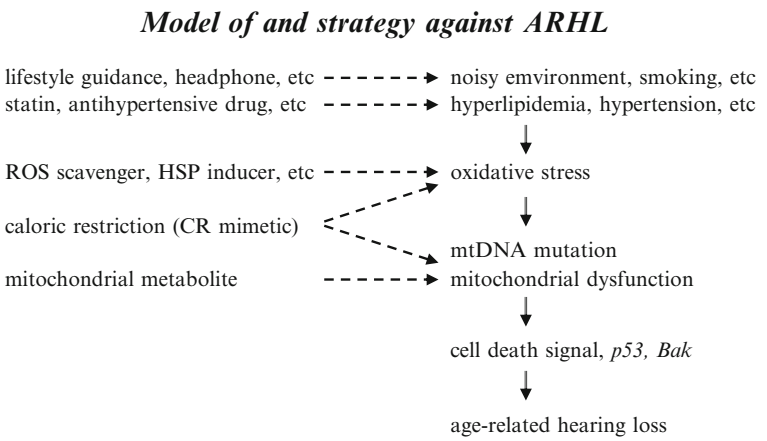

Fig. 16.1 Conceptual model of and strategy against the development of age-related hearing loss

duration of treatments, and species and strain selection. Defining these factors and those we've yet to identify is one of the goals in future research. It is also important to examine if other interventions can prevent/slow ARHL.

In humans, the beneficial effect of dietary and supplement intakes of antioxidants against the development of ARHL has not been well established. On the other hand, dietary intake of antioxidants and PUFA and statin use has appeared to be effective to delay ARHL. In humans, genetic investigation has identified several putative associating genes, including those related to antioxidant defense system and atherosclerosis. Exposure to noise is known to induce excess ROS generation in the cochlea (see Chap. 8 by Yamashita) and cumulative oxidative stress can be enhanced by relatively hypoxic situations resulting from the impaired homeostasis of cochlear blood supply due to atherosclerosis, which could be accelerated by genetic and comorbidity factors. This may explain why PUFA and statin drugs are effective in prevention against ARHL.

The conceptual model for the development of ARHL and strategy to slow/prevent it in humans is shown in Fig. 16.1. It is essential to avoid unnecessary exposure to intense noise to minimize ROS generation in the cochlea. Treatment and prevention of pathological conditions, such as hyperlipidemia, hypertension, and diabetes, by taking statins, antihypertensive drugs, etc., and cessation of smoking in order to prevent atherosclerosis will reduce risk factors for presbycusis. Dietary intake of antioxidants and omega-3 PUFA and restricted intake of high-fat diet may also be beneficial. The evidence of the protective effects of supplementing antioxidants or mitochondria metabolites and restricting total calorie intake against ARHL is limited, or missing, in humans. Large clinical trials are therefore needed to investigate if these interventions can delay or prevent ARHL. It will also be interesting to examine if (aerobic) exercise, which is known to provide beneficial effects systemically, can delay the onset of ARHL.

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Part VII
Hereditary Hearing Loss

Chapter 17
Genes and Hearing Loss: Relationship to Oxidative Stress and Free Radical Formation

David Kohrman

Abbreviations

ARHL	Age-related hearing loss
CAT	Catalase
CR	Caloric restriction
CRISPR/Cas	Clustered regularly interspaced short palindromic repeat/Cas9 endonucleases
ER	Endoplasmic reticulum
ES	Embryonic stem
GWAS	Genome-wide association studies
H_2O_2	Hydrogen peroxide
HPA	Hypothalamic–pituitary–adrenal axis
HSFs	Heat shock transcription factors
HSPs	Heat shock proteins
IDH2	Mitochondrial isocitrate dehydrogenase 2
Mt-Tr	Mitochondrial tRNA arginine gene
NAT2	*N*-Acetyltransferase 2 gene
NF-κb	Nuclear factor kappaB
NGS	Next-generation sequencing
NIHL	Noise-induced hearing loss
Redox	Reduction–oxidation
ROS	Reactive oxygen species
Sirt	Sirtuin

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SNPs	Single nucleotide polymorphisms
SOD	Superoxide dismutase
TALENs	Transcription activator-like effector nucleases
UPR	Unfolded protein response

17.1 Introduction

While reactive oxygen species (ROS) have been implicated as signaling molecules in a variety of normal cellular processes (for review, see Holmstrom and Finkel 2014), overproduction of these by-products of metabolism can result in deleterious oxidation of cellular DNA, proteins, and lipids and lead to cellular dysfunction and/or death (reviewed in Chaps. 1–3). A large body of biochemical, molecular biological, and genetic evidence supports the involvement of reduction–oxidation (redox) imbalance and oxidative stress in hearing loss, including mechanisms that lead to loss following ototoxic agents such as noise (see Chaps. 7–9) and drugs (see Chap. 10–12) and also in age-related hearing loss (ARHL; see Chaps. 13–16). This chapter will summarize genetic evidence from humans and from animal models that implicates oxidative stress-related pathways in hearing loss. This chapter will also suggest relevant future studies in light of recent advancements in genetic and genomic approaches. Genetic studies have provided evidence for the involvement in hearing of genes with direct effects on redox balance and ROS detoxification as well as genes that, following mutation, appear to indirectly alter redox homeostasis through activation of cellular stress pathways. Several of these studies are consistent with a key role of mitochondria as both a source of excessive ROS production and as a mediator of stress signals that regulate apoptotic pathways in the cochlea. Finally, genetic studies also suggest that pathways that are impacted by redox regulation play a role in the normal development of cochlear function.

17.2 Targeted Mutations in Mouse

A number of genes with known roles in intrinsic pathways that metabolize ROS have been implicated in redox homeostasis in the inner ear through analysis of targeted mutations in mouse. These include genes encoding enzymes that reduce a variety of ROS molecules to less reactive compounds, such as superoxide dismutase (SOD) variants: *SOD1* and *SOD2*, which encode two related proteins, the cytosolic Cu/Zn-SOD1 and the mitochondrial SOD2. SOD1 and SOD2 proteins catalyze the reduction of superoxide ion to hydrogen peroxide (H_2O_2). Mice with targeted mutations that decrease or eliminate SOD1 exhibited small elevations of hearing thresholds in young mice and exacerbated hearing loss in older animals, which was

associated with increases in hair cell and spiral ganglion cell loss (McFadden et al. 1999a, b). SOD1 mutants also exhibited slightly higher permanent threshold shifts relative to control mice in response to broadband noise (Ohlemiller et al. 1999). Although this loss of function study supports a role for SOD1 activity in maintaining auditory function during aging and after noise stress, overexpression of SOD1 appears to offer little if any additional protection to hearing function during aging (Keithley et al. 2005), or in response to aminoglycosides (Kawamoto et al. 2004), and in fact worsens the threshold shifts following noise exposure (Endo et al. 2005). This latter response has been attributed to the likely increased levels of H_2O_2 resulting from SOD1 overexpression, the toxic effects of which may not be handled by endogenous levels of other ROS metabolizing enzymes such as catalase.

Hearing in null mutant (−/−) *Sod2* mice has not been evaluated, as these mice die soon after birth with defects in muscle and neural tissue typical of humans with mitochondrial disease (Li et al. 1995; Lebovitz et al. 1996). Although Sod2 +/− heterozygote mice exhibit higher levels of at least one marker of oxidative damage in the cochlea (8-hydroxydeoxyguanosine), mutants exhibit hearing ability comparable to control +/+ mice early and later in life (Le and Keithley 2007; Kinoshita et al. 2013). In contrast to SOD1 overexpression, the overexpression of SOD2 via adenovirus delivery was shown to partially protect guinea pigs against aminoglycoside-induced hearing loss (Kawamoto et al. 2004), suggesting that the mitochondrial enzyme may play a more critical role in combating oxidative damage following ototoxic insult. Targeted knockout of the *Gpx1* gene, which encodes an isoform of glutathione peroxidase, one of a large number of antioxidant enzymes which utilize the small tripeptide glutathione, results in an increase in sensitivity to noise (Ohlemiller et al. 2000). Based on analysis in livers of the knockout mutant, the *Gpx1* gene encodes most if not all of the glutathione-dependent peroxidase activity in mitochondria (Esworthy et al. 1997), supporting the importance of mitochondrial redox status in the cochlea.

17.3 Genetic Control of Quantitative Aspects of Hearing in Mice

Identification of naturally occurring genetic variants that influence quantitative aspects of hearing in inbred mouse strains has also implicated oxidative stress regulation in the auditory system. Characterization of these variants has highlighted the influence of genetic interactions on the extent and onset times of hearing loss (Johnson et al. 2006; Noben-Trauth and Johnson 2009). Similar interactions undoubtedly play a role in the variability in hearing function in humans and contribute to the complexity of the genetic basis of noise-induced hearing loss (NIHL) and ARHL (Friedman et al. 2000).

17.3.1 Cdh23 *and* Sod1

The initial studies of *SOD1* mutant mice were performed on mice with mixed genetic backgrounds (CD1, 129, and C57BL/6) that are known to carry the hypomorphic *Cdh23*^{753A} allele (hearing loss "susceptible") at the *ahl* locus, which is strongly associated with an early onset, high-frequency hearing loss (Johnson et al. 1997, 2000; Noben-Trauth et al. 2003). The alternative *Cdh23*^{753G} allele (hearing loss "resistant") is associated with lower auditory thresholds into later life. *Cdh23* encodes a large cadherin protein that is a component of the inter-stereocilia linkages early in bundle formation (Michel et al. 2005) and of tip links in the developing and mature bundle (Kazmierczak et al. 2007). The *Cdh23*^{753A} allele is associated with altered splicing of the *Cdh23* mRNA, which is expected to result in increased production of a protein lacking one of the multiple extracellular calcium-sensitive ectodomains (Noben-Trauth et al. 2003). In contrast, more severe mutations in *Cdh23* underlie nonsyndromic hearing loss and Usher syndrome in humans (Bolz et al. 2001; Bork et al. 2001) and profound hearing loss and vestibular dysfunction in mice (Di Palma et al. 2001). Evaluation of the effects of *Sod1* loss in a more uniform genetic background indicated substantial progressive hearing loss across all tested frequencies in *Sod1*−/− mutant mice that carried the *Cdh23*$^{753G/G}$ *ahl-resistant* alleles (Johnson et al. 2010). Hearing loss in these mice began by 6 months of age and was associated with minimal hair cell loss, suggesting more subtle functional effects on hearing under conditions of decreased antioxidant activity. *Sod1*+/+ mice that carried the *Cdh23*$^{753A/A}$ *ahl*-susceptible alleles exhibited a progressive hearing loss, mainly at higher frequencies and with a later onset that was associated with significant hair cell loss (Johnson et al. 2010). Defective tip links in these mice are likely to underlie the hearing loss and hair cell death, similar to *salsa* mutants that carry a missense substitution in a CDH23 extracellular domain (Schwander et al. 2009). Mice that were deficient in both *Sod1* (−/−) and *Cdh23* (*Cdh23*$^{753A/A}$) exhibited more substantial threshold elevations, greater than that predicted by additive effects of each mutation. The higher relative expression levels of antioxidant genes in the cochleae of *Cdh23*$^{753A/A}$ mice suggest that tip link defects may increase the ROS burden in hair cells (Staecker et al. 2001). The decrease in antioxidant capability in *Sod1*−/− may thus underlie this synergistic interaction.

17.3.2 *Mitochondrial* tRNA-Arg

Reciprocal backcrosses from three inbred strains (A/J, SKH2/J, Nod/LtJ, which carry the sensitive *Cdh23*^{753A} alleles) with the CAST/Ei strain (*Cdh23*^{753G} alleles) indicated a maternally inherited genetic element carried by the A/J strain that increased thresholds of the *Cdh23*^{753A} homozygous progeny by an additional 15 dB (Johnson et al. 2001). Sequencing of the mitochondrial genomes of these and related strains demonstrated a strong association of this maternal effect with a single

nucleotide insertion within the D-loop region of the mitochondrial tRNA arginine gene (*Mt-Tr*) in the A/J strain. This change is likely to alter the tertiary structure of the tRNA and in turn may affect formation of a charged aminoacyl-tRNA and subsequent translation in the mitochondria of A/J mice. This mitochondrial insertion is reminiscent of mitochondrial variants identified in humans that predispose to hearing loss (Kokotas et al. 2007). These variants have been identified in mitochondrial tRNA and in rRNA genes and have been associated with both nonsyndromic deafness and with a variety of syndromic disorders in humans that include degeneration of neural and muscle tissue in addition to hearing loss. The biochemical effects of these variants on translational fidelity and efficiency in the mitochondria and subsequent respiratory chain defects and on redox imbalances and ROS production are closely associated with downstream functional deficits in cochlear cells (Guan et al. 2000; Hobbie et al. 2008). A variety of apoptotic signaling pathways have been implicated in the cell death that is also associated with mitochondrial dysfunction and permanent hearing loss (Op de Beeck et al. 2011).

17.3.3 Citrate Synthase

Genetic mapping studies in the A/J strain were successful in identifying an additional locus (*ahl4*) that, in conjunction with the original *ahl* locus, contributes to an earlier onset of high-frequency hearing loss in this strain (Zheng et al. 2009). Within the genetic candidate region, a single nucleotide substitution within the coding region of the citrate synthase gene was identified in A/J mice, which is expected to change an evolutionarily conserved histidine residue to asparagine (H55N) (Johnson et al. 2012). The citrate synthase enzyme is localized within the mitochondrial matrix where it catalyzes a rate-limiting step of the citric acid cycle. Although the effects of this variant on mitochondrial energy metabolism have not been directly evaluated, the authors of this study point out that yeast mutants that carry a null mutation in the citrate synthase gene exhibit a higher susceptibility to stress-induced apoptosis associated with excess ROS production (Lee et al. 2007). Reciprocal mating studies also indicated an additive effect of the *Mt-Tr* insertion variant noted above on the extent of early threshold elevation in the A/J strain (Johnson et al. 2012).

17.4 Protective Pathways

Evidence for several endogenous genetic pathways that appear to protect the function of the inner ear has come from gene expression studies following noise and other stresses as well as in the aging cochlea (e.g., Gratton et al. 2011; Tadros et al. 2014). The known output effectors of these pathways include increases in antioxidant molecules as well as decreases in inflammatory cytokines and pro-apoptotic molecules (for review, see Altschuler et al. 2002). Additional insight into protective

pathways has come from animal model studies in which low levels of stress such as heat (Yoshida et al. 1999), noise (Yoshida and Liberman 2000), restraint (Wang and Liberman 2002), and drugs (Ohlemiller et al. 2011) are capable of protecting the inner ear against subsequent more severe ototoxic challenges, a phenomenon known as "conditioning." These conditioning stresses activate both systemic protective mechanisms and pathways that are local to the cochlea. Noise, heat, and restraint stress activate production of glucocorticoids from the adrenal gland via the hypothalamic–pituitary–adrenal axis (HPA), which bind to glucocorticoid receptors on multiple cell types in the cochlea and activate responsive target genes (for review, see Canlon et al. 2007 and Meltser and Canlon 2011). The ability of glucocorticoid receptor agonists such as dexamethasone to at least partially mimic the protection of stress preconditioning and glucocorticoid antagonists to block preconditioning effects supports a crucial role for this system in protection. An effect on target gene transcription by the activated glucocorticoid receptors is presumed to mediate cochlear protection, in conjunction with other transcription factors such as nuclear factor kappaB (NF-κb). An increase in production of the antioxidant glutathione appears to be one of the effectors of this pathway and likely plays a role through its ability to counteract redox imbalances and damaging oxidation effects following ototoxic insults (Nagashima and Ogita 2006). Clarification of mechanisms that underlie conditioning should come from further study of the *Zbtb16* gene, which encodes a transcription factor also known as PLZF. Expression of *Zbtb16* in the cochlea is upregulated by corticosteroids, restraint stress, and noise exposure, and mice homozygous for a loss of function mutation in the gene exhibit a progressive hearing loss (Peppi et al. 2011). In addition, mice that carry the Zbtb16 mutation in heterozygous form are not protected from NIHL by prior restraint conditioning.

Experiments indicating that only the exposed (unplugged) ears of guinea pigs treated with a preconditioning noise, and not unexposed (plugged) ears, exhibited resistance to threshold increases by a subsequent noise challenge suggested that additional protective pathways are also activated locally in the cochlea by stress (Yamasoba et al. 1999). Recent expression and genetic mutant studies are consistent with a local neuroendocrine system involving corticotropin-releasing hormone and cognate receptors that may participate in such an intrinsic system (for review, see Basappa et al. 2012).

A second locally acting protective system involves activation of the classic heat shock response. The heat shock response is driven by induction of the transcriptional activity of heat shock transcription factors (HSFs) that increase expression of a wide range of effector proteins, collectively known as heat shock proteins (HSPs). Several HSPs are induced under stress conditions in the cochlea, including HSP72 following hypoxia (Myers et al. 1992), noise (Lim et al. 1993), hyperthermia (Dechesne et al. 1992; Thompson and Neely 1992; Yoshida et al. 1999), and ototoxins (Oh et al. 2000). Substantial expression of other HSPs, including HSP25 (Leonova et al. 2002), HSP32 (Fairfield et al. 2004), and HSP110 (Yamamoto et al. 2009) has also been observed in the cochlea under stressed and/or unstressed conditions. Maximal HSP induction in the cochlea by heat or noise stress is dependent

upon the transcription factor HSF1 (Gong et al. 2012). Individual HSPs exhibit functions that can directly counteract the higher levels of ROS associated with ototoxic stresses (e.g., the antioxidant activity of HSP32); they can repair or clear proteins that are damaged by oxidation (HSPs 25, 47, 70, and 90—protein chaperone and refolding activities); and they can block pro-apoptotic protein activities that are often activated by excessive oxidative stress (HSPs 27, 70, and 90) (for review, see Kalmar and Greensmith 2009).

Heat shock pathways that are induced by stress, genetic, and pharmacological approaches have been demonstrated to protect a variety of cell types and tissues from a wide range of cellular insults and disease (Akerfelt et al. 2010). Upregulation of HSPs in animal models, either by prior exposure to a stress such as whole body heat or in a transgenic animal with increased HSP70 expression, provides the inner ear with protection from a subsequent exposure to noise or ototoxins (Yoshida et al. 1999; Paz et al. 2004; Takumida and Anniko 2005; Cunningham and Brandon 2006; Taleb et al. 2008, 2009). Protection from this pathway appears to be intrinsic, as local heat activation of the cochlea in vivo also increases resistance to noise damage (Sugahara et al. 2003) and exposure of utricle cultures to heat ameliorates hair cell loss induced by aminoglycosides and by cisplatin (Cunningham and Brandon 2006). Loss of heat shock pathways in mouse models with a knockout mutation of *Hsf1* or of *Hsp70* increases susceptibility to NIHL and noise-induced loss of sensory cells (Sugahara et al. 2003; Fairfield et al. 2005) or to ototoxicity from cisplatin or aminoglycosides (Taleb et al. 2008). *Hsp70* and *Hsp40* were among the genes significantly upregulated in the noise-resistant strain 129/SvEv relative to a noise-susceptible C57BL/6J strain, consistent with a potential role in the resistance phenotype (Gratton et al. 2011).

Recent work from the Cunningham laboratory has supported a non-cell autonomous role for HSP70 in protection of vestibular hair cells from aminoglycosides (May et al. 2013). This group demonstrated that increased expression of HSP70 by supporting cells, induced either by heat shock or by recombinant adenovirus delivery, could diminish hair cell loss following aminoglycoside exposure. Utricle coculture experiments indicate that secreted HSP70 is the bioactive form and may exert its effect by interacting with a cell surface receptor on hair cells. Identification of this putative receptor and the presumed downstream signaling pathways activated by HSP70 binding are likely to suggest new strategies for protection against ototoxins. This study adds to the large list of critical roles played by supporting cells during development and at maturity to maintain function of hair cells and neurons (for review, see Wan et al. 2013).

Additional pathways are involved in activating transcription of *Hsp* genes, as expression of HSPs can be induced even in the absence of HSF1 albeit often at reduced levels (Gong et al. 2012). The triterpene compound celastrol has been shown to protect vestibular and cochlear hair cells from aminoglycoside toxicity (Francis et al. 2011). Celastrol's protective activity appears to be through induction of *Hsp32* expression, which occurs even in an *Hsf1* null mutant background, and is associated with inhibition of JNK-mediated apoptosis. Celastrol may induce *Hsp32* transcription by posttranslational activation of NRF2, a transcription factor previously implicated in *Hsp32* regulation (Trott et al. 2008; Francis et al. 2011).

Other quality control processes that involve redox homeostasis also appear to be critical for maintenance of cochlear function. For instance, recent studies have indicated pathological synergy from redox imbalances and protein misfolding that can occur during formation of disulfide bonds as proteins are processed and trafficked through the endoplasmic reticulum (ER; for review, see Malhotra and Kaufman 2007). Excessive protein misfolding in the ER can result in a stress response known as the "unfolded protein response" (UPR) that includes inhibition of translation and degradation of aberrantly folded proteins. This ER stress has been linked to an increase in ROS production, either directly or through effects on associated mitochondrial dysfunction (Cao and Kaufman 2014). ER stress and excessive ROS production may then exacerbate one another and increase the likelihood of cellular pathology and apoptosis. This synergistic pathology may have relevance to the loss of sensory cells that is a secondary consequence of hearing loss caused by many monogenic deafness disorders. Recent studies in zebrafish have demonstrated that proteins encoded by genes associated with Usher syndrome preassemble into complexes within the ER (Blanco-Sanchez et al. 2014). Mutations in these Usher genes resulted in ER protein trafficking defects and subsequent ER stress associated with apoptosis of sensory cells. ER stress may also play a role in pathology associated with ototoxic exposures of the cochlea, as a marker of ER stress was identified in outer hair cells following systemic injection of kanamycin in mice (Jiang et al. 2006).

An effector pathway in the UPR is detection and degradation of misfolded proteins in the ER by ubiquitin ligase complexes. The *Fbxo2* gene encodes an F-box protein that is a member of these complexes and that interacts with client misfolded glycoproteins (Yoshida et al. 2005). This protein was originally known as OCP1 and is present at high levels in the organ of Corti and brain (Thalmann et al. 1997). Targeted mutation of *Fbxo2* results in progressive hearing loss associated with degeneration of sensory and supporting cells in the cochlea and demonstrates a critical role for this stress response system in long-term maintenance of cochlear function (Nelson et al. 2007).

Decreasing the overall level of nutrients consumed ("caloric restriction"; CR) has been strongly associated with extended lifespan and tissue health in a large number of different organisms, including mammals (for review, see Heilbronn and Ravussin 2003). The association with CR of decreases in oxidative damage in cellular biomolecules has suggested that CR acts by augmenting the defense and/or repair from such damage. Loss and gain of function studies of sirtuin (*Sirt*) genes, which encode NAD^+-dependent deacetylases, have suggested a causative role in at least some aspects of CR through apparent alterations in the expression and/or function of cellular proteins via deacetylation, including those with antioxidant activities (for review, see Donmez and Guarente 2010). CR of 25 % was recently demonstrated to alleviate the threshold shifts, oxidative damage, and sensory cell death in the cochleae of C57BL/6 mice at 12 months of age, compared to littermates fed a normal diet (Someya et al. 2010). CR did not have these protective effects in *Sirt3* null mutants, suggesting that the mitochondrial SIRT3 protein may activate antioxidant defenses in response to CR. The activity levels of mitochondrial isocitrate dehydrogenase 2 (IDH2) activity correlated with increased protection, consistent

with a model in which SIRT3-mediated deacetylation of IDH2 augmented its production of NADPH and, in turn, promoted regeneration of glutathione-based antioxidant activities. Although the effects of CR and *Sirt3* effects on ARHL were not assessed at later ages, this study suggests that *Sirt*-mediated pathways also influence maintenance of cochlear function. Note that the protective effects of CR appear to be strain and species dependent, as similar studies have also demonstrated detrimental effects of CR on long-term auditory function (e.g., Willott et al. 1995). At least one additional *SIRT* family deacetylase gene (*SIRT1*) is expressed in both the cochlea and in the central auditory system and is upregulated by CR (Someya et al. 2007; Xiong et al. 2014).

17.5 Human Genetic Studies

17.5.1 Monogenic Hearing Loss

In addition to the human mitochondrial disorders mentioned above that are due to moderate to highly penetrant mutations in the mitochondrial genome, additional monogenic disorders in humans that include hearing loss have also been linked to defects in genes implicated in oxidative stress and/or associated deleterious effects.

Loss of function mutations in the *MSRB3* gene were recently found to underlie the recessive nonsyndromic hearing loss disorder *DFNB74* (Ahmed et al. 2011). *MSRB3* encodes a protein with methionine sulfoxide reductase activity (Weissbach et al. 2002) and is localized to sensory and supporting cells in the organ of Corti (Ahmed et al. 2011; Kwon et al. 2014). While studies in cultured cells are consistent with an influence on ER and oxidative stress resistance (Kwak et al. 2009, 2012), the lack of an overt accumulation of aberrantly oxidized proteins in the cochlea of mice carrying a targeted deletion of *MSRB3* suggests that a more subtle effect on redox status may underlie the stereocilia and hearing defects in these mutants (Kwon et al. 2014).

Monogenic hearing disorders in humans also suggest an important role for the ER stress response as an important control of ROS formation in cochlear function. Wolfram syndromes 1 and 2 are rare, multisystem disorders that include diabetes, optic atrophy, and hearing loss (for review, see Rigoli and Di Bella 2012). Recessive mutations in the genes *WFS1*, encoding the wolframin protein, and *WFS2*, encoding the Miner1 protein, underlie this complex disorder. In addition, dominant mutations in *WFS1* underlie low-frequency, progressive hearing loss in patients carrying defects at the nonsyndromic *DFNA6/14/38* locus on chromosome 4p16 (Bespalova et al. 2001). Wolframin is an integral membrane protein localized to the ER and lacks clear evidence of known catalytic domains. A recent stem cell model of pancreatic β cells demonstrated that loss of WFS1 was associated with an increase in ER stress markers and defects in processing and secretion of insulin (Shang et al. 2014). Miner1 is an integral membrane protein localized to the ER, and a loss of function mutation that alters its oxidoreductase activity results in ER stress associated with

an activated UPR, loss of Ca^{2+} stores in the ER, mitochondrial dysfunction, and an increase in the level of ROS and RNS (Wiley et al. 2013). Treatment with the antioxidant *N*-acetylcysteine partially alleviated the ER and mitochondrial defects in this cell culture model of *WFS1* loss.

Finally, the relevance of mitochondrial dysfunction to ARHL has been recently demonstrated by identification of a dominant mutation in the *SMAC/DIABLO* gene in humans with progressive hearing loss (Cheng et al. 2011). *SMAC/DIABLO* encodes a mitochondrial protein with pro-apoptotic properties; the dominant mutation is associated with defects in mitochondrial membrane potential (Cheng et al. 2011). This functional data is consistent with recent evidence for roles of pro-apoptotic signaling proteins in mitochondrial homeostasis (Kilbride and Prehn 2013).

17.5.2 Association Studies

A large number of genetic association studies have been performed to identify DNA variants that underlie increased sensitivity to NIHL or ARHL in humans (for reviews, see Van Eyken et al. 2007b, Sliwinska-Kowalska and Pawelczyk 2013, and Yamasoba et al. 2013). These have typically been performed as candidate studies in which the frequencies of allelic variants within or near candidate genes, typically single nucleotide polymorphisms (SNPs), are compared between cohorts with relatively normal hearing versus those with some level of deafness. Alternatively, hearing can be treated as a quantitative trait, often by converting frequency-specific hearing thresholds to a Z-score relative to international age- and gender-specific values (Fransen et al. 2004). To accurately determine the risk attributable to associated variants, these studies must also take into account additional variables in the study populations that are known to affect hearing loss risk, including age, sex, noise exposure, and other comorbidities such as smoking and atherosclerosis (Van Eyken et al. 2007b). Many of these studies have been underpowered, with evaluation of relatively small cohorts of cases and controls. Several associations, however, have been replicated in at least two larger, independent populations and have provided statistical support for the involvement with auditory phenotypes of several genes involved in oxidative stress responses. A missense polymorphism in the *N*-acetyltransferase 2 gene (*NAT2*), which is involved in detoxification of cytotoxic compounds and of ROS, was significantly associated with ARHL in a European cohort (Van Eyken et al. 2007a) and in an independent Turkish cohort (Unal et al. 2005). The associated polymorphism lowers stability of the NAT2 protein (Hein 2002).

Similar studies have been performed in groups that have been exposed to high levels of occupational noise to detect genes that may be associated with differing sensitivities to NIHL. A coding SNP resulting in a missense substitution in the *HSP70* gene was associated with an increased susceptibility to NIHL in independent populations of workers exposed to occupational noise (Konings et al. 2008). In this case, the associated variant appears to also increase circulating HSP70 levels (Afzal et al. 2008). Noncoding SNPs within the catalase (*CAT*) gene were similarly associ-

ated with NIHL in independent populations (Konings et al. 2007). Consistent with a role for catalase-mediated detoxification of ROS during oxidative stress, overexpression of this gene via recombinant adenovirus transfer resulted in increased levels of catalase in the cochleae of guinea pigs and in protection against aminoglycoside ototoxicity (Kawamoto et al. 2004).

SNPs within introns of the *SOD2* gene have been associated with a moderately increased risk for NIHL susceptibility in three independent Italian (Fortunato et al. 2004), Chinese (Liu et al. 2010), and Taiwanese (Chang et al. 2009) cohorts of factory workers. Different SNPs were associated with noise risk in each population: two intronic SNPs (IVS3-23T/G and IVS3-60T/G) in the Italian and Taiwanese studies and rs4880 (−9T>C) in the Chinese study. Although the intronic SNPs appear unlikely to affect SOD2 gene function, the −9T>C polymorphism results in a V16A missense change in the signal peptide of SOD2 that is associated with altered mitochondrial enzyme activity. Note that in each of these cases, the associated SNPs may not directly confer the higher risk of hearing loss, as other SNPs nearby on the chromosome that are in linkage disequilibrium may instead be the causative variants. Further fine mapping and functional studies are required for confirmation of the involvement of these oxidative stress genes in human auditory dysfunction.

17.6 Current and Future Prospects

A number of recent advancements in genomic and genetic technologies and approaches promise to accelerate investigation of the molecular mechanisms that underlie function and pathology in the auditory system, including the relationship of redox regulation and free radical formation to these processes. These include advances in genotyping and sequencing strategies to increase the efficiency and success of human and mouse forward genetic studies for identification of novel genes involved in disease, as well as improvements in the methods used to create mutant models in animals and in cell culture and to evaluate the pathological effects of the mutations.

17.6.1 Human Genetic Studies

The advent in recent years of high-throughput genotyping arrays for simultaneous evaluation of large numbers of human genetic variants (>100,000) has driven genome-wide association studies (GWAS) to investigate complex traits (for review, see Stranger et al. 2011). This permits an unbiased approach to identify variants that alter susceptibility to these traits and has been used to search for genetic contributions to ARHL (Huyghe et al. 2008; Friedman et al. 2009; Van Laer et al. 2010) and also for variation within the range of normal auditory function (Girotto et al. 2011).

Identification of variants associated with hearing deficits, despite their likely small individual effects on risk, may prove very helpful in identification of molecular pathways that suggest novel therapeutic treatments of auditory disorders. Genotype information from large numbers of individuals is required for sufficient power to detect the low relative risk conferred by most single variants. The high genetic heterogeneity underlying highly penetrant, monogenic hearing loss in humans suggests similar heterogeneity is also likely to be associated with more complex risk. Recent GWAS analyses of hearing loss have analyzed genetically isolated populations in an attempt to reduce this complexity and to take advantage of more extensive linkage disequilibrium across the genome that is likely to be present in these populations and to increase the power of the association studies (Van Laer et al. 2010; Girotto et al. 2011). Growing knowledge of human genetic variation provided by large-scale, full genome sequencing is providing a rich source of potential variants, including those that are much less common (<5 % population frequency). A number of bioinformatic approaches have been used to predict the effects of protein-coding variants on gene function, including evolutionary conservation and structural assessments (Cooper and Shendure 2011). A variety of experimental studies, such as chromatin immunoprecipitation and RNA deep sequencing, have contributed to annotation of predicted cis-acting regulatory elements and noncoding RNAs in the much larger nonprotein-coding portions of the genome (Gerstein et al. 2012). GWAS will undoubtedly become more focused on direct detection of causative variants rather than neutral variants that are in linkage disequilibrium, as the catalog of potentially deleterious variants in the human genome increases (Willer and Mohlke 2012).

Evidence to support a causative role of an associated variant on disease risk ultimately requires further testing to verify effects on gene expression and/or function and on phenotype, ideally in an animal or cell culture model system. Recent advances in techniques to make precise changes in the genome have increased the efficiency and decreased the costs involved in creating targeted mutations and other genome manipulations. These techniques make use of sequence-specific DNA endonuclease enzymes (TALENs, transcription activator-like effector nucleases, and CRISPR/Cas, clustered regularly interspaced short palindromic repeat/Cas9 endonucleases) to generate double-stranded DNA cuts or nicks that stimulate cellular DNA repair and recombination at locations of interest in the genome (for review, see Gaj et al. 2013). TALENs derive sequence specificity from engineering the DNA binding domains of transcriptional activator proteins as part of a hybrid endonuclease, while the CRISPR/Cas systems depend upon interaction of the Cas nuclease with a specific guide RNA that includes sequences complementary to the genomic target. These recombineering systems provide the ability to introduce specific genetic variants directly into the genome of mice, zebrafish, and other model organisms without the expensive and time-consuming need for embryonic stem (ES) cells that are required in traditional genome targeting approaches. The newer nuclease approaches could, for example, be used to test the functional consequences of candidate variants that are identified in human cohorts associated with predispositions to premature hearing loss or higher susceptibilities to ototoxic agents such as

noise, aminoglycosides, or cisplatin. CRISPR/Cas and TALEN targeting techniques may also be used to introduce epitope tags into the genomic locus of genes of interest to permit, for example, in vivo analysis of protein localization and protein–protein interactions (Yang et al. 2013).

Advances in cellular reprogramming and directed differentiation strategies to generate specific auditory system cell types from undifferentiated cells and from other cells such as fibroblasts also offer promising opportunities to study genetic pathways and mechanisms involved in inner ear dysfunction and responses to ototoxic stress. These strategies make use of the regulated expression of specific transcription factors as well as growth factor and other media supplements to alter epigenetic control of cell fate and differentiation pathways (Buganim et al. 2013). Although the strategies to date remain relatively inefficient in terms of cell yield, a number of laboratories have been successful in generating cells with molecular and functional phenotypes resembling sensory hairlike cells from ES cells and from terminally differentiated cells such as fibroblasts from mouse and human (e.g., Koehler et al. 2013; Ouji et al. 2013; Ronaghi et al. 2014). Similar studies have been used to generate neuronal cells with characteristics of spiral ganglion neurons (e.g., Tong et al. 2010; Nayagam et al. 2012). Successful reprogramming of cells from humans carrying highly penetrant Mendelian mutations or lower penetrance risk variants should permit in-depth analysis of pathways associated with inner ear dysfunction in a cellular context that better approximates conditions in vivo. For instance, this approach would have the potential to address questions regarding the effects of different nuclear and mitochondrial genetic backgrounds on oxidative stress pathways and offer a platform for high-throughput drug screens for efficacy in ameliorating ROS toxicity or redox dysfunction.

17.6.2 Next-Generation Sequencing

Next-generation sequencing (NGS) refers to a collection of newer technologies that permit the simultaneous sequencing of millions of nucleotides using high-throughput DNA synthesis and detection strategies (for review, see Ansorge 2009). NGS approaches have been successfully applied to expression studies of transcript abundance (RNAseq) that provide sensitivities equal to or greater than typically found in traditional array-based approaches (for review, see Morozova and Marra 2008). RNAseq also permits direct identification of novel transcripts and alternatively spliced isoforms and can be used to more fully investigate the downstream consequences on gene expression of, for instance, stress conditioning approaches in the cochlea. Such studies should broaden our knowledge of relevant pathways and provide additional targets for protective therapies. RNAseq approaches also have utility to better understand the mechanisms involved in mutant models of hearing loss, especially those that involve defects in transcription factors. A recently generated genetic model of auditory neuropathy is a prime candidate for such studies. FOXO3 is a transcription factor previously implicated in response to a range of stresses,

including oxidative, in many tissues (for review, see Maiese et al. 2009). The *Foxo3* gene is expressed in the cochlea and FOXO3 protein is translocated to the nucleus in spiral ganglion cells upon low-intensity sound exposure, suggesting a role in noise stress (Gilels et al. 2013). Targeted deletion of this gene resulted in progressive hearing loss associated with subtle structural changes in the synapses of inner hair cells but without evidence of cell loss (Gilels et al. 2013). Evaluation of the predicted transcriptional changes will be necessary to determine if expression of oxidative stress-related genes is altered in the cochlea by the *Foxo3* mutation. In addition, an increased sensitivity of heterozygous carriers to NIHL would support a protective role for the gene in stress-induced hearing loss.

NGS has also been applied to the study of epigenetic regulation, through strategies that identify changes in chromatin and DNA modifications that influence gene expression (Boyd-Kirkup et al. 2013). These strategies will be very useful to correlate changes in these modifications with hearing loss due to ototoxic stress and aging and to investigate a potential causal role in pathogenic mechanisms.

NGS of human genomes, in addition to providing a more complete assessment of overall genetic variation and candidate variants that underlie complex traits, has also greatly accelerated the identification of high penetrance mutations in Mendelian disorders, including those that include inner ear dysfunction (for reviews, see Rabbani et al. 2012 and Boycott et al. 2013). Whole genome or whole exome sequencing of affected individuals from pedigrees too small for linkage studies and from sporadic cases with no familial history of disease has provided compelling evidence for a large number of causative mutations and has essentially supplanted traditional positional cloning and targeted sequencing of candidate genes. Whole genome sequencing, together with other genomics approaches such as array comparative genomic hybridization (array CGH), has also highlighted the involvement of variations in the copy number of genes and genomic regions in human disease (for reviews, see Alkan et al. 2011 and Girirajan et al. 2011). These copy number screens have recently identified a novel large deletion in individuals with profound nonsyndromic hearing loss that appears to contain important regulatory elements for the nearby connexin genes, *GJB2* and *GJB6* (Wilch et al. 2010). Similar studies of individuals with inherited progressive hearing loss led to identification of a large inverted genomic duplication of 270 kb in size that includes the *TJP2* gene, which encodes a tight junction protein (Walsh et al. 2010). The upregulation of a number of pro-apoptotic genes associated with overexpression of TJP2 may be the basis for sensory dysfunction in this family.

17.6.3 Proteomics

Newly developed proteomics strategies that combine mass spectroscopy and chemical or genetic tagging of reactive cysteine residues should be helpful in the identification of potential targets of ROS-mediated signaling and of chaperone

pathways implicated in protection (for reviews, see Leonard and Carroll 2011 and Thamsen and Jakob 2011). For instance, the lack of a general increase in aberrantly oxidized proteins in the cochlea of mice lacking the methionine reductase MSRB3 suggests that changes in the redox status of a more limited set of putative target proteins may underlie the developmental defects and hearing loss in these mutants (Kwon et al. 2014).

17.6.4 Mouse Models

In addition to the new targeted mutations that will be made possible by the genome engineering approaches detailed above, several other mouse research tools and approaches should prove useful in the coming years to provide additional insight into the genetics of hearing loss. An international collaborative effort, begun in 2007, to generate mutations in each of the approximately 20,000 protein-coding genes in the mouse genome has continued to provide valuable models for biomedical research, including models relevant for investigation of inner ear biology and genetics (International Mouse Knockout Consortium et al. 2007). Several targeting vectors have been used in this effort, including vectors that are designed to produce both standard knockout alleles (complete loss of function in all cells) and conditional knockouts alleles (Skarnes et al. 2011). The conditional alleles can be bred to a growing panel of mouse strains that express sequence-specific recombinases such as Cre or Flp, activities of which are under the control of specific ligands and/or regulatory elements to permit control over the timing and cell type specificity of gene deletion events in the inner ear (Yu and Zuo 2009). These conditional alleles may therefore be useful to investigate a variety of more refined research questions, such as those related to the identity of vulnerable cell populations and the temporal requirements and nature of putative protective pathways with respect to ototoxic stresses. Researchers using these targeted lines for auditory studies must, however, keep in mind that most of the lines have been generated on a C57BL/6-derived genetic background, which is predisposed to an early onset, high-frequency hearing loss (Johnson et al. 1997) and is susceptible to NIHL (Davis et al. 2001). Moving the targeted mutation through successive backcross breeding into an alternative strain such as CBA or FVB with more robust auditory function throughout life will be necessary in some cases.

A number of breeding strategies are being used to more readily untangle the genetic variation that underlies complex traits in the mouse. Many of these strategies have generated new hybrid inbred strains that harbor selected regions (congenic strains) or full chromosomes (consomic strains) from one strain in the background of another strain. These types of strains have greatly assisted in identification of novel genetic variants associated with ARHL (Nemoto et al. 2004; Johnson et al. 2012). Similar approaches should prove useful to characterize the genetic basis of known strain differences in auditory function, including differences

in responses to noise stress (Yoshida et al. 2000), protection from conditioning stresses (Barden et al. 2012), and aging (Noben-Trauth and Johnson 2009; Noben-Trauth et al. 2010; Schacht et al. 2012).

A large ongoing effort has generated a large set of recombinant inbred strains, known as the "collaborative cross," that are composed of a patchwork combination of genomes derived from eight inbred strains (Threadgill et al. 2011). Through collective study by many laboratories, these strains provide the opportunity to investigate the genetic basis of phenotypic variation in a host of developmental and physiological processes across lifespan as well as to test for potential common genetic control points among these processes (Aylor et al. 2011). Finally, analysis of progeny from a cross involving four inbred strains of mice has recently been used to localize several novel ARHL loci (Schacht et al. 2012). These "four-way" progenies exhibit several features that are useful as model systems for analysis of complex genetic traits in mice, including their heterozygous nature across the genome that may better approximate the characteristics of human genomes (Miller et al. 1999).

17.6.5 Therapeutic Strategies

The discovery of loss of function mutations that predispose to hearing loss, as well as pathways that provide protection against ototoxic stresses, has suggested a number of therapies for prevention or amelioration of hearing loss (see Chaps. 4, 9, 12, 16). These interventions have typically been designed to replace or augment dysfunctional or overburdened endogenous pathways that have been implicated in protection against oxidative and other stresses or to block downstream apoptotic programs. Approaches to induce effector proteins of the heat shock response such as HSP70 are instructive regarding the promise and likely difficulties in translating basic science discoveries to the clinic. A number of agents, including celastrol (Francis et al. 2011), geldanamycin (Yu et al. 2009), and geranylgeranylacetone (Mikuriya et al. 2005, 2008), that induce the activity of various HSPs have shown efficacy in reducing the extent of sensory cell dysfunction associated with aminoglycoside toxicity, noise overexposure, and aging. However, significant toxicity has been observed in vivo or in cultured cells with each of these agents, likely from off-target effects and/or from disruption of cellular homeostasis due to abnormal activation of their intended targets (for review, see Neef et al. 2011). In addition, given the increased levels of HSPs that have been observed in a variety of tumors and their correlation with poor prognosis, care must be taken with the potential use of such agents with respect to hearing loss (for review, see Ciocca and Calderwood 2005). As with any therapeutic approach, more precise targeting to critical parts of cellular pathways and optimization of dose–response characteristics and tissue specificity will be required to move these agents into the clinic. Additional investigation of the genes and pathways implicated in hearing loss and the use of animal and tissue culture model systems will provide better insight into the most appropriate cellular targets and important platforms for preclinical testing.

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Chapter 18
Strategies for the Treatment of Hereditary Hearing Loss

Glenn Green and Yehoash Raphael

18.1 Introduction to Hereditary Hearing Loss

Hereditary hearing loss may affect any site along the auditory pathway including the ossicular chain, the tectorial membrane, the hair cells, and the auditory nerve. The hearing loss may be conductive, sensorineural, or a combination of both. It may be isolated hearing loss (nonsyndromic with no associated medical problems) or part of a constellation of medical problems affecting other organs in the body (syndromic). Hereditary hearing loss may be apparent early on, even before language has developed (prelingual), or not develop until old age (presbycusis) (Angeli et al. 2012; Bovo et al. 2011). Additionally, genetic contributions play a key role in susceptibility to key environmental causes of deafness: aminoglycoside exposure, noise exposure, and otitis media (Emonts et al. 2007; Sliwinska-Kowalska and Pawelczyk 2013; Konings et al. 2009; Rahman et al. 2012). Hereditary hearing loss may be caused by a mutation in a single gene (monogenic) or the interactions of multiple genes with the environment (polygenic and complex disorders).

Our understanding of the specific genes involved in hearing loss has burgeoned with the completion of the human genome and improvement in gene mapping techniques. Most of this new knowledge is focused on single-gene disorders. For this reason, we will focus here on the most common single-gene disorders.

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Despite this focus, similar principles apply to complex, multifactorial hearing loss, including those associated with presbycusis (see Chap. 14) and noise-induced hearing loss (see Chap. 8). The delineation of the specific genes behind complex disorders will require large epidemiologic and genetic studies although smaller-scale studies have already yielded interesting insights (Konings et al. 2009).

18.2 Diagnosis of Hereditary Hearing Loss

Congenital severe-to-profound deafness affects approximately 0.1 % of children in the United States. In most of these families, there is no history of hearing loss. Genetic studies have shown that more than half of prelingual deafness in developed countries is genetic (Morton and Nance 2006). Most of this hearing loss is autosomal recessive and nonsyndromic. Genetic mapping has identified dozens of genes for nonsyndromic dominant, recessive, X-linked, and mitochondrial hearing loss (http://hereditaryhearingloss.org, Hilgert et al. 2009). Dozens of additional genes associated with syndromic causes of hearing loss have been identified. Multigenic inheritance patterns are much more difficult to untangle.

The most common type of hereditary hearing loss varies based upon the population (see Table 18.1). Yet, across most populations worldwide, the most frequently identified genetic mutations causing hearing loss are mutations in one of three genes: *GJB2* (the gene encoding connexin 26), *SLC26A4* (the gene encoding pendrin), and *MTRNR1* (the mitochondrial gene encoding 12S rRNA) (Hilgert et al. 2009; Wang et al. 2011). The etiology of hearing loss in each of these entities has been attributable in part to oxidative stress and free radical formation as discussed below.

Standard evaluation for hearing loss includes a detailed medical history, physical examination, and audiometry. Imaging with MRI or CT scan may be useful for many individuals. With the discovery of dozens of genes for dominant and recessive hearing loss, genetic tests have become increasingly effective in identifying the specific cause of hereditary hearing loss. For all children with congenital hearing loss, genetic tests are now the first diagnostic modality recommended by otolaryngologists (Chan et al. 2011; Hart and Choo 2013; Shearer and Smith 2012).

Table 18.1 Most common causes of hereditary hearing loss

Gene	Protein	Other names	Common mutations	Associated features
GJB2	Connexin 26	DFNB1	35delG, M34T, 167delT, V37I, 235delC	Rarely skin conditions
SLC26A4 (*PDS*)	Pendrin	DFNB4	His723Arg, Thr416Pro, Leu236Pro	Mondini deformity, euthyroid goiter
MT-RNR1	12S rRNA		A1555G	Susceptibility to aminoglycosides

18.3 Role of Free Radicals in the Most Prevalent Forms of Hereditary Hearing Loss

Free radicals play an important role in the pathogenesis of each of the three most common forms of hereditary hearing loss. Previously, the causal mechanisms behind hereditary hearing loss were largely unknown. The key to greater understanding of the pathogenesis of hereditary hearing loss has been the identification of the specific genes involved. The same genes can subsequently be examined in mouse models. Through these mouse models, the role for oxidative stress in hereditary hearing loss has been identified.

Still, free radicals should not be misconstrued as playing a role in the pathogenesis of *all* forms of hereditary hearing loss. For example, individuals with mutations in proteins forming the tectorial membrane typically have midfrequency hearing loss that is stable (McGuirt et al. 1999; Verhoeven et al. 1998). Mutations that decrease the exposure of the hair cells to sound may protect against noise-induced hearing loss (Legan et al. 2005).

18.3.1 GJB2 *(Connexin 26)*

Mutations in the gene encoding connexin 26, *GJB2*, are the most common cause of genetic hearing loss and account for over half of hereditary congenital deafness in many populations. The *GJB2* mutation 35delG is the most common mutation in Europe, North America, South America, Northern Africa, and India (Mahdieh and Rabbani 2009). In Eastern Asia, the *GJB2* mutation 235delC is the most common (Yan et al. 2003). 167delT is the most common mutation seen among the Ashkenazi Jews and in Palestinians (Sobe et al. 1999). Each of these mutations is common within the specified population and has a founder effect where a single mutation spread broadly throughout a population (Yan et al. 2003; Lerer et al. 2001; Ohtsuka et al. 2003; Van Laer et al. 2001). This lends support to a selection advantage for carriers of these mutations. Missense mutations in *GJB2* are also very common, especially M34T, V37I, and L90P (Snoeckx et al. 2005). Hundreds of hearing loss mutations have been described in comprehensive databases and include both missense and nonsense mutations. Over 3 % of the hearing population in the United States is a carrier of a mutation in connexin 26 that causes deafness (Green et al. 1999).

The hearing loss associated with *GJB2* mutations may vary from mild to profound (Snoeckx et al. 2005). The hearing loss is typically symmetric and worse in the higher frequencies (Green et al. 2003). *GJB2* mutations are the most common etiology of profound congenital sensorineural hearing loss. However, many individuals have been identified who have passed newborn hearing screens, indicative of, at worst, mild hearing loss, who were subsequently found to be deaf (Green et al. 2000; Norris et al. 2006). The degree of hearing loss is worse for truncating and nonsense mutations than missense mutations (Snoeckx et al. 2005). In childhood

and adulthood, the hearing loss is typically stable, but may be progressive (Kenna et al. 2010; Chan et al. 2010; Hochman et al. 2010). Neither improvement in hearing nor fluctuation has been noted with *GJB2* mutations (Green et al. 2003).

The hearing loss associated with *GJB2* mutations is typically nonsyndromic. However, several dominant mutations in *GJB2* are associated with skin abnormalities (Lee and White 2009). The mutation causes no neurologic or cognitive dysfunction apart from direct consequences of its effect on the cochlea.

GJB2 mutations are known to interact with mutations in the gene *GJB6*, which encodes connexin 30 and is also found in the cochlea (Lerer et al. 2001). Additionally, there appears to be an interaction between mutations in *MTRNR1* and *GJB2* (Kokotas et al. 2010).

The gene *GJB2* (gap junction beta 2) is found on the long arm of chromosome 13 (13q11-q12). This gene encodes connexin 26 (Cx26), one of several connexins found within the ear. Connexins aggregate in groups of six around a central 2.3 nm pore to form a connexon. Connexons from adjoining cells can covalently bond to form a channel. In large aggregates these channels form plaques and gap junctions. These channels allow the transmission of small ions and signaling molecules. They are presumed to be important in the potassium pathway recirculation where potassium entering the hair cells during mechanotransduction is shuttled back to the stria vascularis. A similar pathway can enable shuttling of free radicals.

Mutations in *GJB2* reduce the ability of supporting cells to shuttle potassium, free radicals, and other metabolic by-products involved in sound transduction (Martínez et al. 2009). Cell death is seen in the hair cells and supporting cells in both human temporal bones and mouse models. The mechanism for this apoptotic cell death appears to be increased production of free radicals and potassium-induced cytochrome c release. In addition, there may be a direct dependence of free radical formation on potassium channel function during the respiratory chain in mitochondrial function. Local extracellular accumulation of potassium due to the absence of connexin 26 could result in both increased glutamate concentrations and oxidative stress. Trauma-induced decreases in connexin 26 levels in the spiral ligament can be prevented by pretreatment with the antioxidant tempol or the nitric oxide synthase inhibitor $N(\omega)$-nitro-L-arginine methyl ester (L-NAME) (Nagashima et al. 2009). Gap junction channels are under negative regulation by reactive oxygen species (Martínez et al. 2009). Overexpression of connexin 26 is known to have a protective effect against oxidant-induced death in a nonotologic epithelial cell line (Hutnik et al. 2008). Antioxidants may protect gap junction function from oxidative damage induced by ototoxic insults (Fukuda et al. 2000).

18.3.2 SLC26A4 *(Pendred Syndrome and Isolated Enlarged Vestibular Aqueduct)*

Mutations in the gene encoding *SLC26A4* are the second most common causes of nonsyndromic hearing loss and the most common cause of syndromal hearing loss in many populations (Albert et al. 2006). The carrier rate for *SLC26A4* mutations in

the hearing population is lower than *GJB2*, and there is no evidence of a founder effect or selective advantage for mutations in this gene. Several mutations including H723R, T416P, and L236P have been seen across geographically diverse locations (Campbell et al. 2001). Interestingly, many individuals with only a single identified mutation in *SLC26A4* have hearing loss (Albert et al. 2006).

The hearing loss associated with *SLC26A4* mutations is typical of that associated with enlargement of the vestibular aqueduct, viz., progressive and fluctuating. Hearing loss is predominantly sensorineural with a conductive hearing loss in the lowest frequencies. Hearing loss may be asymmetric. Hearing may precipitously decline after minor head trauma or progress slowly over time (Honings et al. 2008). Progression to profound hearing loss during adolescence is common for individuals with mutations in *SLC26A4*.

Mutations in *SLC26A4* are associated with inner ear abnormalities that can be visualized with computed tomography or magnetic imaging of the temporal bones (Campbell et al. 2001; Everett et al. 1997). The Mondini deformity is the most common abnormality and consists of enlargement of the vestibular aqueducts with incomplete partition of the cochlea. However, isolated vestibular aqueduct enlargement and even unilateral vestibular aqueduct enlargement are often seen in individuals with *SLC26A4* mutations. Enlargement of the vestibular aqueduct is alternatively called large vestibular aqueduct, enlarged vestibular aqueduct, or dilated vestibular aqueduct. Precise diagnostic criteria remain in flux, but a vestibular aqueduct with a width that is greater than 1.0–1.5 mm is a relatively broadly accepted criterion (Boston et al. 2007; Vijayasekaran et al. 2007). In individuals with unilateral vestibular aqueduct enlargement, hearing loss may occur bilaterally or unilaterally. Unilateral hearing loss may occur on the side contralateral to the enlarged vestibular aqueduct.

Most hearing loss associated with *SLC26A4* is nonsyndromic. However, mutations may occur with euthyroid goiter formation that begins in the late teens (Everett et al. 1997; Pryor et al. 2005). Hearing loss when associated with goiter is known as Pendred syndrome. Pendred syndrome is the most common cause of syndromal hearing loss. The likelihood of having or developing thyroid disease may be correlated with the genotype (Pryor et al. 2005; Campbell et al. 2001). Thyroid abnormalities can be identified prior to the development of goiter through a perchlorate discharge test which identifies a partial organification problem.

Mutations in *SLC26A4* are known to interact with mutations in the potassium channel gene *KCNJ10* to cause deafness (Yang et al. 2009). Many individuals, largely with enlarged vestibular aqueducts, have been identified with hearing loss and only a single mutation in *SLC26A4* (Campbell et al. 2001; Pryor et al. 2005).

The gene *SLC26A4* (solute carrier family 26 anion exchanger, member 4) is found on the long arm of chromosome 7 (7q31). This gene encodes pendrin. Pendrin is a transmembrane anion transporter that transports chloride, iodide, and bicarbonate (Scott et al. 1999). Pendrin is present in the kidneys, inner ear, and thyroid. Within the inner ear, the role of pendrin is thought to be due to chloride and bicarbonate exchange. In the thyroid gland, the effect of iodine transport disruption leads to goiter formation.

Mutations in *SLC26A4* result in disruption of the inner ear. Initially, the anatomic entity of enlarged vestibular aqueduct was presumed to be the cause of hearing loss. Evidence against this being the case includes hearing loss in the side contralateral to the enlarged vestibular aqueduct and the failure of surgical intervention directed at the vestibular aqueduct. When the gene was identified as an anionic transporter, a metabolic basis for hearing loss was reconsidered. The creation of animal models and precise dissection of the metabolic derangements associated with *SLC26A4* mutations has identified oxidative stress as a strong role player in the development of deafness (Hochman et al. 2010). Loss of the bicarbonate secretion leads to acidification of the endolymph and subsequent free radical stress-mediated loss of *Kcnj10* in the stria vascularis which abolishes the production of the endocochlear potential (Singh and Wangemann 2008). For a detailed discussion of the stria vascularis and endocochlear potential, see Chap. 3 by Ohlemiller.

18.3.3 MTRNR1 *(the Mitochondrial Gene Encoding 12S rRNA)*

Mutations in the mitochondrial gene *MTRNR1* are the most common cause of mitochondrial deafness (Hutchin and Cortopassi 2000). The gene was first identified based upon hereditary predisposition to deafness after therapeutic doses of streptomycin were given for antituberculosis therapy (Prezant et al. 1993). A single mutation, A1555G, is a leading cause of deafness in many populations worldwide. This mutation has recurred multiple times across ethnic backgrounds (Torroni et al. 1999). This mutation is a leading cause of deafness around the world and is estimated to account for a large portion of all hearing loss associated with aminoglycosides. In some countries, this mutation is very common, particularly in familial hearing loss of late onset (Estivill et al. 1998).

The hearing loss associated with *MTRNR1* mutations varies from congenital profound to mild and is often not seen until late adulthood (Estivill et al. 1998). However, with even small doses of aminoglycosides, profound hearing loss may result (Estivill et al. 1998). For a detailed discussion of the role of the A1555G mutation in aminoglycoside-induced hearing loss, see Chap. 10 by Rybak and Brennar.

The hearing loss associated with *MTRNR1* mutations is typically nonsyndromic, but has associations with hypertension or cardiomyopathy (Santorelli et al. 1999; Chen et al. 2012). The T1095C mutation in *MTRNR1* may result in isolated hearing loss or deafness, neuropathy, and parkinsonism (Thyagarajan et al. 2000; Zhao et al. 2004).

MTRNR1 mutations have been shown to interact with *TFB1M* (the mitochondrial transcription factor B1) which is a modifier for the severity of hearing loss in patients with A1555G (Bykhovskaya et al. 2004). The gene *MTO1*, involved in tRNA hypermodification, has been shown to play a role in the development of hearing loss in individuals with A1555G mutation who have not been exposed to aminoglycosides (Li et al. 2002).

The gene *MTRNR1* is found in mitochondrial (648–1,601 bp) rather than nuclear DNA. Outside of the nucleus of a cell where the paternally and maternally inherited DNA from the 23 pairs of chromosome subsides, several copies of DNA are present within the mitochondria. Mitochondrial DNA found within eukaryotes is different in many ways than nuclear DNA and is postulated to have an ancient bacterial origin (Fischel-Ghodsian 1999). This DNA is inherited usually exclusively from one's mother from the mitochondria of the egg. The copies of mitochondrial DNA within a cell may be entirely the same (a condition called homoplasmy) or may differ (a condition called heteroplasmy). A1555G is usually found in homoplasmy.

The gene *MTRNR1* encodes the 12S rRNA. 12S rRNA is a component of the small mitochondrial ribosome subunit (28S).

The folding of this gene, specifically the stem-loop structure, is altered in the A1555G mutation such that there is a great similarity between the human 12S rRNA and the corresponding ribosomal RNA (16S rRNA) found in bacteria that are sensitive to aminoglycosides (Perletti et al. 2008; Prezant et al. 1993). This altered folding pattern allows aminoglycosides to bind to the human-altered mitochondrial ribosome in a way similar to the way that it binds to the bacterial ribosome. The binding of the aminoglycoside to the human ribosomal RNA results in destruction of the mitochondria.

Mitochondrial defects preferentially adversely affect the inner ear in comparison to other tissues. The effectiveness of free radical scavengers, both endogenous (like glutathione levels) and exogenous, in preventing aminoglycoside hearing loss further points to the role of free radicals in the development of this type of hearing loss. At the cellular level, aminoglycosides cause damage through excessive formation of reactive oxidative species (Talaska et al. 2006). These in turn trigger cell death pathways through caspases or cathepsins.

18.4 Interventions for Hereditary Hearing Loss

For individuals with free radical-associated hearing loss that is associated with genetic mutations, there are two specific medical goals: to optimize performance and to maintain or improve hearing. Ideally, we will do this with as little risk and as few side effects as possible. Through understanding of the specific causes of deafness, diagnostic and prognostic information is gained. This can specifically lead to individualized therapeutic programs (Table 18.2).

18.4.1 Behavioral Interventions

It is well known that some individuals are particularly sensitive to otologic insults of many types. The genetic basis for some of this susceptibility is beginning to be identified. For example, greater susceptibility to noise-induced hearing loss requires

Table 18.2 Treatment of hereditary hearing loss

Behavioral
Avoidance of specific insults in genetically susceptible populations
Devices
Amplification
Middle ear surgery
Cochlear implantation
Medications
Steroids
Antioxidants
Genetic interventions
Nonspecific (i.e., increased endogenous antioxidant production)
Specific correction of the underlying defect

the analysis of large numbers of individuals (Konings et al. 2009; Sliwinska-Kowalska and Pawelczyk 2013). If the susceptible individuals can be identified, a conscientious decision can be made to avoid exposure.

The clearest implementation of this has been the avoidance of aminoglycosides in individuals that carry the *MTRNR1* mutation A1555G. Screening for this mutation in individuals prior to the administration of aminoglycosides could lead to the prevention of deafness in many individuals (Rahman et al. 2012; Bitner-Glindzicz et al. 2009). A genetic screening program is being initiated at several centers around the world.

For individuals with *SLC26A4* mutations, head trauma can precipitously result in hearing loss (Gopen et al. 2011; Honings et al. 2008). Avoidance of sports and other activities that can result in head trauma is recommended.

Diet modification and supplementation is an intriguing potential intervention for individuals with susceptibility to free radical-induced hearing loss. Diets rich in antioxidants have been associated with improved hearing, as discussed in detail in Chap. 6 by Spankovich. Micronutrient supplementation with fluoride has markedly decreased hearing loss associated with otosclerosis, an often hereditary form of hearing loss of uncertain etiology (Chen et al. 2002; Vartiainen and Vartiainen 1997). Supplementation with biotin has improved another (Straussberg et al. 2000). Therapeutic micronutrients can potentially be identified based upon the genomic profile of an individual, e.g. individuals with mutations in genes associated with otosclerosis may benefit from fluoride supplementation.

18.4.2 Devices

Hearing aids may be useful for many individuals. Generally, hearing aids are much better than cochlear implants for individuals with less than severe hearing loss. Hearing aids are broadly used by individuals with mutations in *GJB2*, *SLC26A4*,

and *MTRNR1*, as well as many other types of genetic hearing loss. For some types of genetic hearing loss (such as those associated with otosclerosis), stapedectomy or other middle ear surgical implants may be of benefit (Chen et al. 2002).

When hearing aids are inadequate, cochlear implantations may be useful. The results of cochlear implantation may vary based upon the genetic etiology of deafness. Specifically, children with *GJB2* mutations obtain exceptional results after cochlear implantation (Green et al. 2002). Adults with *SLC26A4* mutations have excellent results after cochlear implantation (Hochman et al. 2010). Individuals with *MTRNR1* mutations do well after cochlear implantation (Sinnathuray et al. 2003). Not all individuals with genetic mutations will perform well after cochlear implantation: *CHD7* mutations are a known predictor of poor performance after cochlear implantation (Lanson et al. 2007).

18.4.3 Medications

The recognition of oxidative stress as a common pathway underlying many forms of hereditary hearing loss opens up the possibility of personalized interventions through the use of antioxidants and gene therapy.

18.4.3.1 Glucocorticoid Steroids

High-dose oral glucocorticoids have been effective for some causes of hearing loss. Sudden hearing loss associated with enlarged vestibular aqueduct may improve with steroid administration (Lin et al. 2005). The mechanism for glucocorticoids improving hearing is not known, but may in part be due to the function of glucocorticoids in decreasing inflammation and increasing cell survival pathways, including those involved in free radical scavenging (Himeno et al. 2002).

18.4.3.2 Antioxidants (Animal Model)

The use of antioxidants in the treatment of hearing loss has been poorly studied for mutations in *GJB2*, *SLC26A4*, and *MTRNR1*. We have tested *GJB2* conditional knockout mice for the effects of an oral antioxidant and magnesium (ACEMg) diet supplementation. Mice that received the treatment arm demonstrated improved hearing thresholds and hair cell preservation relative to controls. Of importance, mice with a different genetic defect (*Diap3*) demonstrate worse hearing after ACEMg administration for unclear reasons.

Studies demonstrating the efficacy of antioxidants in the treatment of aminoglycoside ototoxicity may have direct relevance for hearing loss associated with mitochondrial mutations. The mouse model for this defect has not been tested for the effects of an antioxidant regimen.

18.4.3.3 Antioxidants (Human)

The use of antioxidants in treating hereditary hearing loss is poorly studied. Use of over-the-counter micronutrients allows for potential examination of the role of antioxidants in hereditary hearing loss. In one case report, a child with *GJB2* mutations and progressive hearing loss had amelioration of his hearing loss with the administration of high doses of antioxidants (Thatcher et al. 2014).

18.4.4 Genetic Interventions

When the genetic etiologies of hereditary hearing loss were first identified, there was great optimism that gene therapy designed to correct the underlying defect may result in elimination of the risk of hearing loss. There are high regulatory burdens for the use of gene therapy in humans after initial problems with its use (Ginn et al. 2013). Safety must be considered relative to the potential benefits of gene therapy.

18.4.4.1 Specific and Nonspecific Gene Therapy (Animal Model)

Gene therapy offers the potential to treat hereditary hearing loss. Many vectors have been developed that can direct expression of the desired proteins within the inner ear (Fukui and Raphael 2013). *BDNF* gene therapy ameliorated spiral ganglion nerve degeneration associated with *GJB2* mutations in a mouse model (Takada et al. 2014). Gene therapy to increase the endogenous production of antioxidants has been efficacious in ameliorating ototoxic hearing loss and is an intriguing therapeutic treatment for hereditary hearing loss that has yet to be tested.

Specific gene replacement of a genetic defect in the mouse was first performed with *VGLUT3* (Akil et al. 2012). Introduction of *SLC26A4* targeted to the endolymphatic sac in Slc26a4 mutant mice results in restoration of inner ear function (Li et al. 2013).

18.4.4.2 Nonspecific Gene Therapy (Humans)

A human trial for gene therapy with *ATOH1* for sensorineural hearing loss was initiated in 2014 (Thomson 2014). This trial is a hallmark toward the eventual application of specific and nonspecific genetic treatment for hereditary hearing loss.

18.5 Conclusion

The genetic factors that lead to hearing loss are being identified. Genomic sequencing of individuals with hearing loss now supplements the traditional medical evaluation of hearing loss. Genetic susceptibility determines the residual hearing after many environmental insults. As we increase our understanding of the specific genes and pathways underlying hearing loss, genome-tailored customized treatment regimens will become possible.

Traditional interventions for hereditary hearing loss have been amplification, behavioral interventions, and cochlear implantation. Medical interventions targeted at oxidative stress may play a future role in the treatment of each of the most commonly identified genes that cause hereditary hearing loss: *GJB2* (connexin 26 deafness), *SLC26A4* (enlarged vestibular aqueduct syndrome), and *MTRNR1* (12S rRNA). Since oxidative stress plays a pivotal role in the pathogenesis of each of these types of hereditary hearing loss, it may prove to be beneficial to decrease the effects of oxidative stress through diet modification, use of antioxidants, or gene therapy. Further studies, especially those that are double blinded and placebo controlled, are needed. Ultimately, exposure and underlying genetic risk profile will determine the differing agents, routes of administration, timing of administration, and dosages.

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Part VIII
Cochlear Implants, Radiation, Trauma and Other Stress Factors

Chapter 19
Loss of Residual Hearing Initiated by Cochlear Implantation: Role of Inflammation-Initiated Cell Death Pathways, Wound Healing and Fibrosis Pathways, and Potential Otoprotective Therapies

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Abbreviations

ABR	Auditory brainstem response
AIF	Apoptosis-inducing factor
Apaf-1	Apoptotic peptidase-activating factor 1
BAX	Bcl-2-associated X protein
BID	BH3 Interaction domain
BMPs	Bone morphogenic proteins
CI	Cochlear implant
Cyt. *C*	Cytochrome *C*
DIABLO	Direct Inhibition of apoptosis-binding protein with low p*I*
DISC	Death-inducing signaling complex
D-JNKI-1	D-JNK inhibitor peptide 1
DR	Death receptor
DR3	Death receptor three
DR4	Death receptor four
DR5	Death receptor five
DXM	Dexamethasone
EAS	Electroacoustic stimulation
EIT	Electrode insertion trauma
EMT	Epithelial to mesenchymal cell transition

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Endo-G	Endonuclease G
EP	Endolymphatic potential
ERK	Extracellular signal regulated kinase
FADD	Fas-associated death domain
GCR	Glucocorticoid receptor
HC	Hair cells
IAPs	Inhibitors of apoptosis
IHC	Inner hair cell
IL-1β	Interleukin one beta
IL-6	Interleukin six
iNOS	Inducible nitric oxide synthase
JNKs	c-JUN N-terminal kinases
L-NAC	L-*N*-acetylcysteine
MAPKs	Mitogen-activated protein kinases
NFκB	Nuclear factor kappa B
NT-3	Neurotrophin-3
OC	Organ of corti
OHC	Outer hair cell
Omi/HtrA2	Mammalian homology of bacterial high temperature requirement protein
PI3K	Phosphatidylinositol 3-kinase
Ppy/pTS/NT-3	Polypyrrole/para-toluene sulfonate containing neurotrophin-3
ROS	Reactive oxygen species
SG	Spiral ganglion
SIBS	Styrene–isobutylene–styrene
Smac	Second mitochondria-derived activator of caspases
tBID	Truncated BID
TGFβ	Transforming growth factor beta
TNF α	Tumor necrosis factor alpha
TNFR1	Tumor necrosis factor receptor 1
TRADD	TNFR1-associated death domain
TRAIL-R1	TNF-related apoptosis-inducing ligand receptor 1
TRAIL-R2	TNF-related apoptosis-inducing ligand receptor 2
TRAMP	TNF-like receptor apoptosis-mediating protein
XIAP	X-linked inhibition of apoptosis proteins

19.1 Introduction

The mammalian cochlea is composed of both supporting and sensory receptor cells which are essential for the proper function of this auditory receptor (Van De Water 2012). When the cochlea is exposed to a high level or even repeated moderate levels of oxidative stress, many of the auditory hair cells (HC) can be damaged beyond their ability to self-repair (Baker and Staecker 2012). As a consequence, there is a loss of input to the central auditory pathways. If this deficit of neural input from the

cochlea is profound, then an alternate source of signal input is required to recreate the lost auditory input. This has led to the development of a device that translates auditory signals into a train of electrical stimuli that can be delivered to the cochlear nerve and spiral ganglion (SG) neurons in a tonotopic fashion. This is accomplished by modern cochlear implants which enable profoundly deaf individuals to hear again (Eshraghi et al. 2012). This accomplishment has been recognized and honored by the Lasker–DeBakey Clinical Medical Research Award to three pioneers in this field of auditory prosthetic devices (i.e., Lasker–DeBakey Clinical Medical Research Award, 2013 to Drs. Clark, Hochmair, and Wilson).

Modern cochlear implants (CI) require deep insertion of a multi-electrode array into the cochlea. This can be accomplished via either a cochleostomy or insertion through the round window membrane into the perilymph-filled cavity (i.e., scala tympani), directly below the basilar membrane and organ of Corti (OC), and adjacent to the SG neurons in Rosenthal's canal (Rask-Andersen et al. 2012) (Fig. 19.1). This procedure frequently results in electrode insertion trauma (EIT), which is the topic of this chapter. In addition to damage to the OC, EIT can damage the nerves that are encased in the bony spiral modiolus, and containing channels (Fig. 19.2) which allow communication between the perilymph and the auditory nerve (Rask-Andersen et al. 2012). This unique anatomical feature (Fig. 19.2) will be important later in this chapter when drug delivery into the cochlea is presented and discussed. Although great strides have been made in the design of atraumatic electrode arrays that reduce EIT, there can still be an inflammatory response within the cochlear tissues after implantation, as well as the generation of an excessive level of free radicals resulting in oxidative stress (e.g., reactive oxygen species; ROS) (Bas et al. 2012).

As reviewed in Chap. 7 by Altschuler, sound pressure waves enter the ear canal and initiate vibrations of the tympanic membrane that are amplified by the ossicular chain of the middle ear. These vibrations travel to the oval window and create fluid shifts that travel through the cochlear partition to vibrate the basilar membrane in a tonotopic manner. The stereocilia of the auditory HCs are then displaced from their resting position, leading to inner hair cell (IHC) membrane depolarization, glutamate exocytosis into the IHC—afferent nerve terminal synaptic clefts, and electrical signaling to the auditory cortex through SG neuron activation. The outer hair cells (OHCs) are the mechanosensory receptor cells of the cochlea that are essential for detecting and amplifying mechanical vibrations of the basilar membrane for normal hearing. Fortunately, CIs can restore hearing when there has been a significant loss of the auditory OHCs and/or IHCs. Electrical signals from a CI bypass the need for these auditory HCs as it directly stimulates the SG neurons in a frequency-specific manner for auditory perception.

Placement of this electrode array into the scala tympani can be physically traumatic in even the most skilled hands and can induce intracochlear expression of various pro-inflammatory and pro-apoptotic factors that can lead to apoptosis of some or even all of the remaining HCs in the cochlea. By preventing programmed cell death of these mechanosensory cells of the OC following implantation, preservation of residual hearing can be achieved postoperatively, which has assumed increasing importance since the current trend is to implant these patients with hybrid

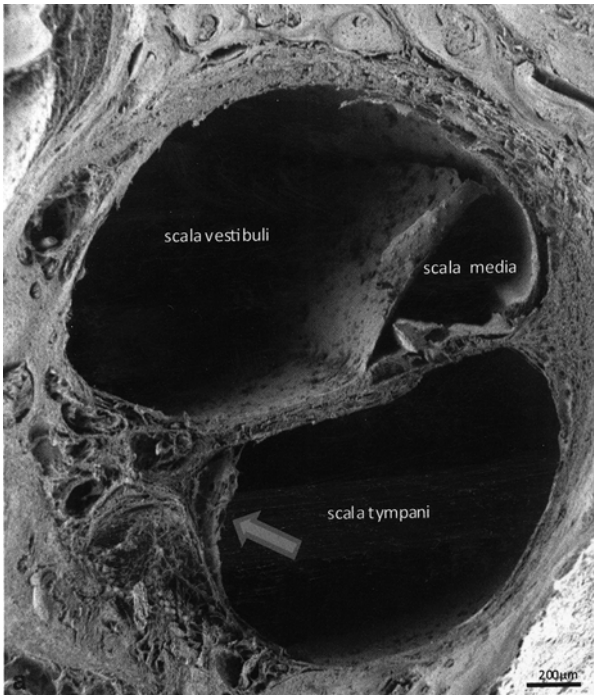

Fig. 19.1 A scanning electron micrograph of the lower basal turn of a human cochlea. The *arrow* indicates the direction of insertion of a cochlear implant electrode array into the scala tympani between the scala tympani fluid space and the spiral ganglion neurons located within Rosenthal's canal is a thin sheet of mesothelium covering the trabeculated bony columns that guide the peripheral axons of these auditory ganglion cells to the sensory hair cells within the organ of Corti. Bar = 200 µm

devices that combine electrical and acoustic stimulation (Turner et al. 2004). Patients that have successfully preserved residual hearing after cochlear implantation can utilize both electric (i.e., from CI stimulation) and acoustic hearing (i.e., from amplified sound wave stimulation of functional HCs in the low-frequency region of the organ of Corti). Acoustic low-frequency hearing may allow patients to better distinguish fine pitch differences that should allow improved speech recognition in noise, melody recognition, and music appreciation (Turner et al. 2004; Gantz et al. 2005, 2006, 2009; Gfeller et al. 2006; Adunka et al. 2010, 2013; Carlson et al. 2011; D'Elia et al. 2012; Cosetti et al. 2013; Santa Maria et al. 2013).

Although low-frequency preoperative hearing thresholds may be preserved shortly after surgery, the auditory HCs may continue to degenerate with time and lead to long-term progressive loss of residual hearing (Santa Maria et al. 2013). This phenomenon may represent a progressive degeneration that originates from the patient's underlying disease process, a prolonged low-level inflammatory process stemming from a foreign body reaction to the electrode array, or very likely a combination of both of these processes. Therefore, it is imperative to understand how cochlear implantation affects apoptosis and non-apoptotic initiated loss of auditory HCs to allow identification of solutions to prevent these processes, to preserve residual hearing long term and improve CI benefits after surgery and in the long term.

Another issue that leads to poor hearing outcomes following cochlear implantation is an increase in electrode impedance secondary to the development of local fibrosis and osteoneogenesis. Wound healing is a complex process that involves the

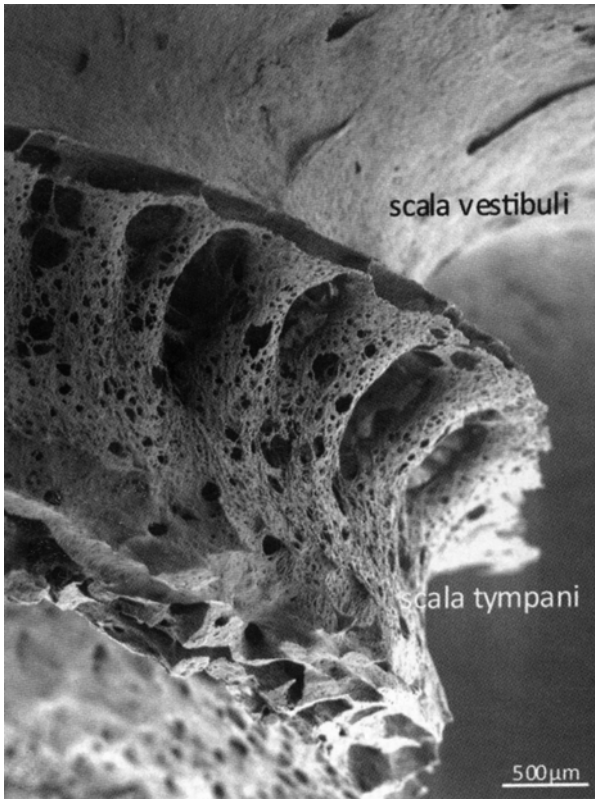

Fig. 19.2 A scanning electron micrograph of the thin trabeculated bony columns that enclose the spiral ganglion neurons that are located within Rosenthal's canal. These are fragile structures easily damaged and also this micrograph depicts the routes of communication between the fluid space of the scala tympani and Rosenthal's canal. Bar = 500 µm

expression of various pro-inflammatory cytokines, cross-communication across many cell types, and modulation by surrounding extracellular and vascular components. By understanding the sequential and cyclic events that occur on a molecular level to transform electrode insertion-initiated inflammation into scar tissue, drugs may be developed to target key events in this inflammatory cascade to slow or halt the progression of cochlear fibrosis and improve post-implantation speech and music perception.

19.2 Cochlear Implantation and Trauma-Induced Loss of Residual Auditory Hair Cells

As reviewed in Chap. 2 by Leeuwenburgh, apoptosis refers to a highly regulated form of programmed cell death that is characterized by cell blebbing and shrinkage followed by chromatin condensation and DNA fragmentation (Hengartner 2000). Apoptosis can occur through two main cell death signaling pathways: the intrinsic pathway (i.e., mitochondrial death pathway) and the extrinsic pathway [i.e., death

receptor (DR) pathway]. Both pathways can independently activate downstream events that lead to apoptosis (caspase-dependent cell death) or apoptosis-like cell death [apoptosis-inducing factor (AIF) or endonuclease G (Endo-G)-mediated cell death]; however, cross-communication can also occur between these two signaling cascades and promote programmed cell death.

In the intrinsic pathway, stress signals, such as oxidative stress from expression of high levels of ROS, can initiate depolarization of the mitochondrial membrane to release intramembranous pro-death proteins into the cytoplasm of an affected cell. These mitochondrial proteins include cytochrome *C* (cyt. *C*), second mitochondria-derived activator of caspases (Smac)/direct inhibitor of apoptosis-binding protein with low p*I* (DIABLO), mammalian homolog of bacterial high temperature requirement protein (Omi/HtrA2), AIF, and Endo-G, cyt. *C*, Smac, and Omi/HtrA2 promote caspase-dependent activation of apoptosis, while AIF and Endo-G initiate caspase-independent pathways of cell death.

Cyt. *C* will bind to apoptotic peptidase-activating factor 1 (Apaf-1) and initiate the formation of an apoptosome, which is an oligomeric complex that can activate caspase-9 (an initiator caspase) to in turn initiate the activation of downstream caspases such as caspase-3 and -7 (downstream executor caspases) to promote cell death (Li et al. 1997). Smac, on the other hand, will bind to X-linked inhibitor of apoptosis proteins (XIAP), thereby disrupting XIAP–caspase interactions and releasing basal inhibition of caspases (e.g., caspases-3, -7, and -9) (Srinivasula et al. 2000). Similar to Smac, Omi/HtrA2 can bind and neutralize inhibitors of apoptosis (IAPs) to promote caspase-dependent apoptosis; however, the effects of Omi/HtrA2 are irreversible through cleavage of IAPs (Yang et al. 2003). AIF and Endo-G do not depend on caspase activation, but rather translocate into the cell nucleus and promote DNA fragmentation and chromatin condensation resulting in caspase-independent apoptosis of the affected cell (Susin et al. 1999; Li et al. 2001).

The extrinsic pathway is also known as the DR pathway as it requires binding of a death ligand to its complementary receptor on the cell membrane to activate caspase-8 and downstream events that lead to apoptosis. Tumor necrosis factor receptor 1 (TNFR1) and Fas are the best characterized receptors of this DR-activated cell death pathway; however, other receptors can also activate this signaling cascade. These receptors include tumor necrosis factor (TNF)-like receptor apoptosis-mediating protein (TRAMP, or DR3), TNF-related apoptosis-inducing ligand receptor 1 (TRAIL-R1, or DR4), and TRAIL-R2 (DR5) (Itoh and Nagat 1993; Tartaglia et al. 1993; Bodmer et al. 1997; Griffith et al. 1999; Chaudhary et al. 1997). When the death ligand binds to its complementary DR, several intracellular death domains containing adaptors, such as Fas-associated death domain (FADD) and TNFR1-associated death domain (TRADD), are recruited along with caspase-8 to form a death-inducing signaling complex (DISC) (Tartaglia et al. 1993; Chinnaiyan et al. 1995; Hsu et al. 1995; Schneider-Brachert et al. 2004). DISC is important for activation of caspase-3 and caspase-dependent programmed cell death. In addition, the extrinsic pathway signaling can promote cleavage of BH3 interacting-domain (Bid) death agonist to its active truncated form, tBid. Active tBid can lead to Bcl-2 associated X protein (Bax)-initiated depolarization of the

mitochondrial membrane, pore formation, and activation of the intrinsic pathway to promote apoptosis (Li et al. 1998).

Cell death can also occur through mechanisms other than apoptosis, i.e., necrosis. Necrosis is a form of cell death that was thought to be unorganized and is characterized by increased permeability of the cell membrane, cellular edema with eventual cell membrane rupture, and release of intracellular contents into the surrounding environment. Recently, necrosis has been renamed "regulated necrosis" because it has been found to depend on specific signaling factors that lead to this morphologic form of cell death (i.e., RIP1 and -3). Stimuli that may initiate necrosis include alkylation DNA damage, excitotoxins, and ligation of DRs (Galluzzi et al. 2012).

Cochlear implantation trauma likely causes auditory HC and residual hearing loss from a combination of apoptosis, apoptosis-like cell death and regulated necrosis; however, currently there are no studies at this time that evaluate the role of regulated necrosis in post-implant loss of a patient's residual hearing or loss of hearing in an animal model of EIT-induced hearing loss.

Cochlear implantation surgery and EIT cause apoptosis of auditory HCs through activation of both the mitochondrial and DR signaling pathways. Several events during cochlear implantation are linked to apoptosis of auditory HCs, and they include: (1) acoustic and vibrational injury from drilling of the ossicles, labyrinth bone, and/or cochleostomies; (2) blood byproducts from inadvertent entrance of blood into the cochlea; (3) displacement of bone dust into the perilymphatic compartments of the inner ear; (4) mechanical injury to the auditory HCs from the insertion of the electrode array; (5) traumatic penetration of the basilar membrane resulting in alterations of endocochlear potential (EP) and a mixing of endolymph with perilymph; (6) fracture of osseous spiral lamina; (7) electrode tip dissection of the spiral ligament and stria vascularis important for cochlear homeostasis; (8) immediate or delayed foreign body reaction; and at times, (9) inflammatory response from bacterial infection acquired during the process of CI.

Attention to various steps of cochlear implantation can help prevent loss of residual auditory HCs and hearing loss. Soft surgical techniques have been described to reduce the trauma and inflammatory response that result in apoptosis, or apoptosis-like cell death or regulated necrosis of auditory HCs following cochlear implantation. In brief, cochlear implantation involves drilling of the mastoid through a retro-auricular incision and identification of the incus and lateral semicircular canals as initial landmarks to guide the drilling of the facial recess. Once the facial recess is drilled, the stapes and promontory are identified and the bony ledge obscuring visualization of the round window is drilled to improve the angle for the insertion of the electrode array. If a cochleostomy is required, drilling will proceed using a small bur bit anterior–inferior to the round window membrane. The CI electrode array is carefully inserted through an incision in the round window membrane or through a pre-drilled cochleostomy using micro-instruments employed and guided under high magnification of an operating microscope. Figure 19.3 illustrates basic human temporal bone anatomy important for safe cochlear implantation.

Techniques of soft surgery in cochlear implantation aim to preserve the survival of auditory HCs important for residual acoustic hearing and are described below

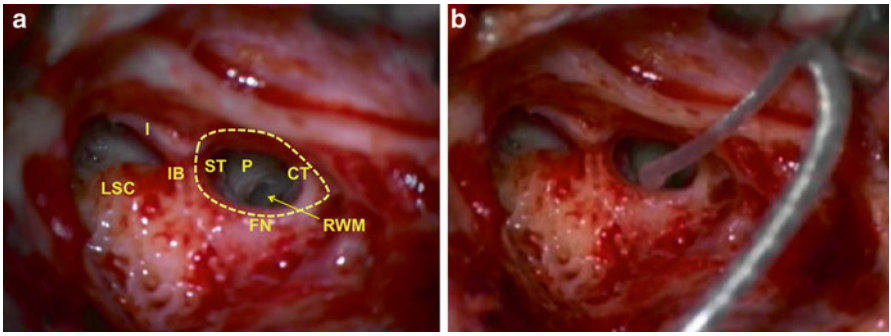

Fig. 19.3 Surgical anatomy for electrode insertion into a right cochlea. (**a**) A right mastoidectomy has been drilled and the lateral semicircular canal (LSC) and incus (I) are identified. These landmarks assist in identifying the incus buttress (IB), facial nerve (FN), and chorda tympani (CT) and safe drilling of the facial recess (*dotted line*). Once the facial recess is drilled, the stapedial tendon (ST) and the bony promontory (P) of the cochlea are visualized. Drilling of the promontory may be necessary to expose the round window membrane (RWM) for successful cochlear implantation or proper drilling of a cochleostomy anterior–inferior to the RWM. (**b**) A cochlear implant electrode is seen inserted through the RWM into the cochlea

(Lehnhart 1993; Cohen 1997; Kiefer et al. 2004; Roberson 2005; Roland et al. 2005; Eshraghi 2006; Skarzynski et al. 2007; Berrettini et al. 2008; Jia et al. 2011; Postelmans et al. 2011; Havenith et al. 2013). The basis of this technique has scientific merit in preventing loss of auditory HCs and hearing loss following cochlear implantation as outlined and highlighted in detail in Fig. 19.4.

Vibrations from drilling of the ossicular chain or on the bony promontory can be transmitted to the inner ear and injure the stria vascularis and auditory HCs. The exact mechanism of vibration-induced inner ear damage is not entirely clear but may involve alterations of the EP via injury to the intermediate cells of the stria and increased permeability of the stria vascularis (Seki et al. 2001; Marcus et al. 2002; Hashimoto et al. 2006). Changes in EP likely affect the homeostasis of auditory HCs leading to activation of the mitochondrial pathway of apoptosis or apoptosis-like cell death, since maintenance of the EP is crucial for the survival of the OHCs (Takeuchi et al. 2000; Teubner et al. 2003; Nouvian et al. 2003; Wangemann et al. 2004). Vibration is also associated with intracochlear expression of TNFα and TNFR1, which together can initiate the DR pathway and downstream signaling that leads to apoptosis of auditory HCs (Zou et al. 2005; Dinh et al. 2008a, b; Haake et al. 2009). Vibrational injury can affect HC viability from the apical to the basal turns of the cochlea and initially affects the outer most rows of OHCs before proceeding to injure the second and then first rows of OHCs located toward the cochlear modiolus. SG neuron density was also reduced following exposure to vibration trauma (Bochnia et al. 2005). Furthermore, vibrations from drilling can initiate hearing threshold shifts in both laboratory animals and humans (Zou et al. 2001; Karatas et al. 2007; Migirov and Wolf 2009; Moussavi-Najarkola et al. 2012). High-frequency vibrations have been shown to be more detrimental to hearing than

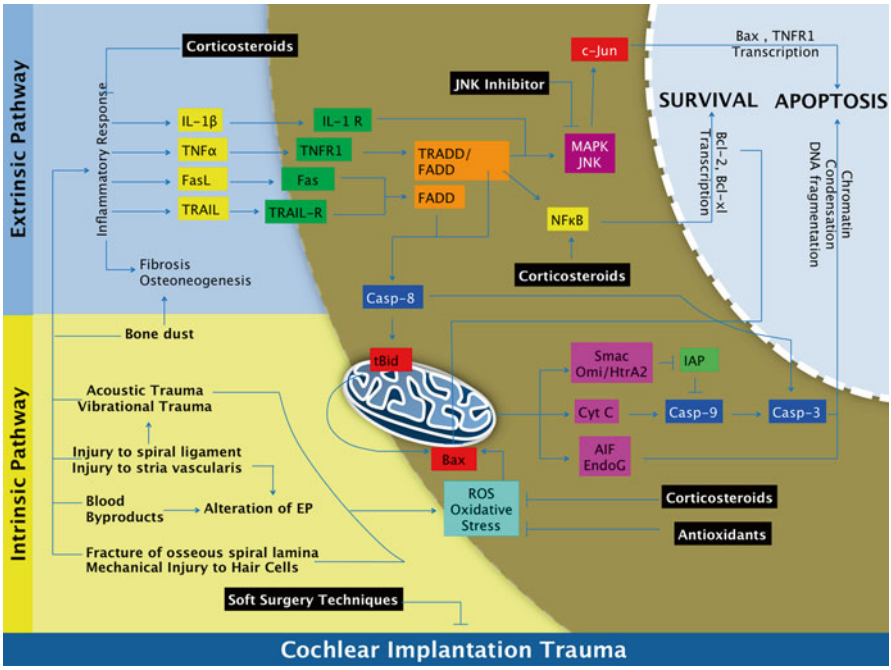

Fig. 19.4 Cochlear implantation and apoptosis. Cochlear implantation can result in acoustic and vibrational trauma, injury to the spiral ligament and stria vascularis, introduction of blood by-products into the cochlea, fracture of the osseous spiral lamina, mechanical injury to auditory hair cells, and alterations in the endocochlear potential (EP) that trigger expression of reactive oxygen species (ROS) and oxidative stress that promote the mitochondrial (or intrinsic) pathway of apoptosis (bottom half of diagram). The intrinsic pathway involves activation of Bax protein and release of mitochondrial proteins that promote apoptosis. Smac, Omi/HtrA2, and Cyt C initiate caspase-dependent apoptosis through activation of caspase-9 and -3, while AIF and Endo-G do not depend on caspases and translocate to the nucleus to initiate chromatin condensation and DNA fragmentation for apoptosis to occur. Cochlear implantation also initiates an inflammatory response and expression of several pro-inflammatory and pro-death cytokines, particularly IL-1β and TNFα. These cytokines trigger a signaling cascade that preferentially activates MAPK/JNK signaling and caspase-8 and -3 that promote the death receptor (or extrinsic) pathway of apoptosis (top half of the diagram). NFκB may also be activated in attempt for cell survival, but to a lesser extent. Caspase-8 from the extrinsic pathway can trigger apoptosis through the intrinsic pathway through tBid activation of Bax activity. Soft surgical techniques, corticosteroids, JNK inhibitors, and anti-oxidants can inhibit pro-death signaling pathways and promote cell survival pathways for auditory hair cell survival

low-frequency vibrations with older animals more susceptible to vibration-induced threshold shifts than younger animals, and surgical drilling with steel cutting burs more likely to affect otoacoustic emissions than drilling performed with diamond burs (Zou et al. 2001; Goyal et al. 2013).

Acoustic trauma represents another mode of injury to auditory HCs during cochlear implantation. Sound emissions from drilling near the cochlea can travel to the sensory receptor tissues through the oval and round windows. Different surgical

drilling burs produce various levels of sound intensity with steel cutting burs producing higher sound pressure levels than diamond burs (Dalchow et al. 2013). Loud noise exposures can injure the stria vascularis, leading to changes in the potassium concentration of the endolymph, alterations in the EP, and damaging levels of oxidative stress (Syka et al. 1981; Wang et al. 1992; Poje et al. 1995; Yamane et al. 1995; Ohlemiller et al. 1999; Ohinata et al. 2000; Hirose and Liberman 2003). The mitochondrial cell death pathways are then triggered, resulting in release of cyt. *C* and caspase-3-dependent cell death of auditory HCs and/or release of Endo-G and AIF responsible for DNA fragmentation and caspase-independent apoptosis (Henderson et al. 2006; Hu et al. 2002; Nicotera et al. 2003; Yamashita et al. 2004; Han et al. 2006). The DR pathway is also activated following acoustic trauma through expression of TNFα and the TNF superfamily of receptors and downstream signaling that also involves activation of the pro-apoptotic mitogen-activated protein kinase (MAPK) signal cascade which can involve activation of the c-Jun N-terminal kinase (JNK) pathway as well as caspase-8-dependent events of programmed cell death (Pirvola et al. 2000; Nicotera et al. 2003; Wang et al. 2003; Hu et al. 2009; Jamesdaniel et al. 2011). In addition, noise-induced injury to the inner ear can cause an acute loss of afferent nerve terminals and delayed degeneration of the cochlear nerve (Kujawa and Liberman 2009). Thus, it is vital to limit the effects of both acoustic and vibration-induced trauma to the cochlea during the process of CI in order to reduce intracochlear apoptotic and apoptotic-like signaling that can interfere with conservation of residual hearing during and after cochlear implantation.

Surgical techniques to minimize bone particles and blood from entering the cochlea will likely reduce the inflammatory burden in the cochlea that can lead to loss of auditory HCs after electrode array insertion. Though there is no clear evidence that bone pâté in the cochlea will cause loss of residual hearing, it can promote osteoneogenesis and fibrosis within the scala tympani that may cause inference with electrode impedance and a delayed electrode array extrusion response (Clark et al. 1995; McElveen et al. 1995). On the other hand, intrascalar blood can lead to temporary and permanent threshold shifts particularly in the apical region when autologous blood is injected into the cochlea of a guinea pig (Radeloff et al. 2007). The hearing threshold shifts are likely a result of erythrocyte degradation which leads to recruitment of inflammatory cytokines (e.g., TNFα, IL-1β, IL-6) and inflammatory enzyme expression [e.g., matrix metalloproteinase-9 (MMP-9) and MAPK], production of ferrous ions leading to excessive levels of ROS production, and changes in EP from potassium spillage into the scala tympani (Van Bergen et al. 1999; Mathiesen et al. 1997; Maddahi et al. 2012). These apoptotic stimuli will activate both intrinsic and extrinsic pathways of apoptosis and apoptosis-like cell death in auditory HCs, thus stressing the importance of avoiding the introduction of blood into the cochlea during the process of CI.

Conservative use of sodium hyaluronate gel during cochlear implantation may help protect residual hearing. Sodium hyaluronate is injected over the endosteum with a micro-lancet prior to penetrating the membrane, but after the bone has been drilled away from the cochleostomy site. This prevents entrance of blood and bone

pâté into the cochlea and prevents perilymph from escaping from the scala tympani prior to electrode array insertion (Laszig et al. 2002). The gel also acts as a lubricant to aid smooth insertion of the electrode array. However, excessive amounts of intracochlear sodium hyaluronate gel can cause auditory HC injury and severe sensorineural hearing loss (Roland et al. 2005). The mechanism of injury is unclear and may involve drug toxicity or mechanical injury from injection that initiates oxidative stress and programmed cell death of auditory HCs. There is no evidence that sodium hyaluronate use alone prevents threshold shifts following cochlear implantation, but it is associated with residual hearing preservation when combined with other soft surgical techniques (Skarzynski et al. 2002; Kiefer et al. 2004; Balkany et al. 2006; Fraysse et al. 2006; Garcia-Ibanez et al. 2009). In addition, suctioning of the perilymph should be avoided during this time to prevent rapid fluid shifts and changes in the endocochlear potential that can lead to oxidative stress and auditory HC programmed cell death; the use of sodium hyaluronate may also act as a small buffer when pericochlear suctioning is unintentional.

The characteristics of an electrode array (i.e., stiff vs. flexible, thin vs. thick, straight vs. contoured), the position of the cochleostomy, the depth of insertion, and their contribution to soft surgical techniques are extremely important. Appropriate selections will aid in smooth electrode insertion parallel to the trajectory of the basal turn, thereby avoiding inadvertent mechanical injury to the auditory HCs, basilar membrane penetration and alteration of EP, fracture of osseous spiral lamina, injury of SG neurons, and dissection of spiral ligament and stria vascularis (Kiefer et al. 2004; Briggs et al. 2005; Gantz et al. 2005; Gstöettner et al. 2006, 2008; Adunka et al. 2007; Baumgartner et al. 2007; Li et al. 2007a, b; Berrettini et al. 2008; Bruce et al. 2011; Helbig et al. 2011a, b; Eshraghi et al. 2012). In addition, intracochlear injuries can lead to osteoneogenesis and fibrosis that can affect electrode impedance (Li et al. 2007a, b; Fayad et al. 2009). The culmination of EIT will not only activate a very robust inflammatory reaction but also result in activation of both the mitochondrial and DR-mediated apoptosis and apoptosis-like pathways to initiate loss of auditory HCs.

Oxidative stress and ROS production from EIT-associated mechanical disruption of intracochlear structures and fluxes in EP can initiate the intrinsic pathway. The mitochondria will then release pro-apoptotic mitochondrial proteins that lead to caspase-3-dependent cell death and Endo-G initiated nuclear condensation, DNA fragmentation, and caspase-independent cell death (Bas et al. 2012; Eshraghi et al. 2013). Expression of pro-inflammatory cytokines (i.e., TNFα and IL-1β) and pro-inflammatory enzymes [inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2)] can also promote production of high levels of ROS and intrinsic pattern of apoptosis (Friedlander et al. 1996; Ho et al. 1998; Nam 2006). TNFα, particularly, can bind to its receptor TNFR1 to initiate DR-associated apoptosis of auditory HCs through activation of MAPK and JNK signaling pathways initiating an upregulation in the expression of pro-apoptotic *Bax* gene and protein expression, upregulation of *TNFR1* gene for apoptosis propagation, downregulation of pro-survival *Bcl-2* and *Bcl-xl* gene expressions, and caspase activity (Dinh et al. 2008a; Eshraghi et al. 2013). As demonstrated, expression of these factors can promote

downstream pathways that act cumulatively to tilt the pendulum from cell survival to cell death, resulting in a progressive loss of auditory HCs and residual hearing at several days post-EIT following a cochlear implantation (Eshraghi et al. 2005). Several therapeutic agents that target particular aspects of the EIT-induced cell death pathways in auditory HCs have demonstrated protection of residual hearing following an EIT event (see Sect. 20.4, below).

19.3 Cochlear Implantation, Inflammation, and Fibrosis Signaling

Cochlear fibrosis and ossification can occur as a result of CI-EIT-initiated inflammatory cascade and can affect efficiency of the CI (Fig. 19.5). Higher levels of current would be necessary to overcome the impedance caused by scar tissue and bone growth (Choi and Oghalai 2005). Targeting the mediators responsible for fibrosis and neo-osteoneogenesis after a CI-EIT insult may improve hearing outcomes following cochlear implantation by reducing intracochlear scar tissue and new bone formation. In the case of device failure, CI reimplantation will be less risky if the cochlear lumen is not obstructed by fibrous tissue and newly formed bone.

The inflammatory response that occurs following CI-EIT is a complex biological process that involves vascular and cellular components as well as soluble substances.

Disruption of blood vessels and leakage of blood components into surrounding tissue can lead to aggregation of platelet cells and a signaling cascade that produces a clot rich in fibrin and other extracellular matrix proteins (Bergmeier and Hynes 2012). Soon after, fibrin is degraded, several enzymatic proteins called the complement system are activated, and various cytokines and chemokines are released locally. This constellation of events leads to the recruitment of immune cells to the wound site and the initiation of the inflammatory phase of wound healing.

Neutrophils, a type of white blood cell, are the first immune cells to arrive following activation of the inflammatory response. They are a major source of pro-inflammatory cytokines, such as interleukin (IL)-1α, IL-1β, IL-6, and TNFα (Mutsaers et al. 1997). Monocytes, another type of immune cell, are attracted to the wound by local expression of chemokines. Exposure to these cytokines released by neutrophils can cause monocytes to differentiate into M1 macrophages, which secrete other cytokines. M1 macrophages also stimulate inducible nitrous oxide synthase (iNOS) activity and expression of ROS and reactive nitrogen species (RNS) that can be detrimental to surrounding cells. Monocytes that reach the clot extracellular matrix proteins can be stimulated by other cytokines (e.g., IL-4, IL-10, and IL-13) and develop into M2 macrophages. M1 macrophages are involved in clearance of injured cells, while M2 macrophages release anti-inflammatory cytokines and promote wound healing and tissue repair (Delavary et al. 2011; Airel and Timor 2013).

M2 macrophages release high levels of growth factors from the TGF-β family of proteins, which activate a fibroproliferative response that leads to wound healing.

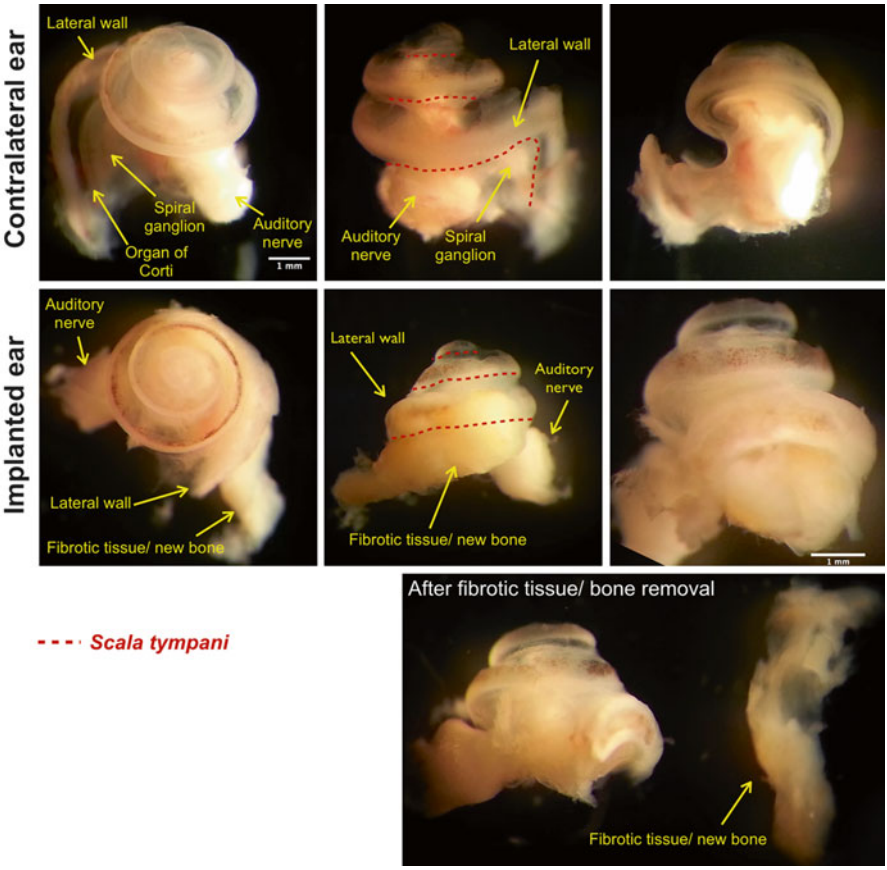

Fig. 19.5 An increase in impedance is well correlated with an increase of fibrotic tissue growth in the scala tympani located in the area of the inserted electrode array. The macroscopic images correspond to a contralateral (not implanted) control cochlea and an implanted cochlea of an adult Norway brown rat. Fibrotic and/or bone tissue are found along the scala tympani, where the electrode insertion trauma occurred. The scala tympani for the three turns of the rat cochlea is located under the *red dashed line*. Bar = 1 mm

There are three isoforms of TGF-β proteins that participate in wound healing. TGF-β1 is associated with fibrocyte proliferation, fibrosis, and osteoneogenesis while TGF-β2 and TGF-β3 are important for scarless wound healing (Li et al. 2006).

In an in vitro model of EIT, there was an early upregulation of gene transcripts of pro-inflammatory cytokines TNF-α and IL-1β in the apical turns of rat OC explants 24 h post-EIT. At a later time point (i.e., 96 h), there was a dramatic downregulation of TNF-α and IL-1β and a significant increase in TGF-β1 gene expression levels (Bas et al. 2012). These changes occur to a lesser extent in the middle and basal turns of these EIT-OC explants. Early expression of TNF-α and IL-1β represent the initial inflammatory response that occurs following EIT and late expression of TGF-β1 is largely responsible for the fibrosis that ensues.

In the same model, increases in TGF-β3 were demonstrated in the middle and basal turns of rat OC explants following EIT, and these levels were magnified by Dexamethasone (DXM) treatment. TGF-β3 plays an important role in scarless wound healing and this increase in TGF-β3 in the middle and basal turn segments may be beneficial in reducing the inflammatory process and encouraging healing at the trauma site with lower levels of fibrotic scar tissue formation (Tandon et al. 2010). These results also suggest that treatment with DXM may encourage wound healing and tissue repair with less fibrosis.

Wnts are a family of proteins that bind and activate serpentine membrane receptors encoded by the Frizzled gene family. There are three main Wnt signaling pathways which include: (1) the Wnt/β-catenin (canonical) pathway; (2) the planar cell polarity (non-canonical) pathway; and (3) the Wnt/Ca^{2+} (non-canonical) pathway. The differential expression of various Wnt proteins and signaling cascades during embryogenesis is involved in cochlear differentiation (Daudet et al. 2002). Both Wnt and TGF-β proteins are important growth factors in the regulation of tissue homeostasis, development, differentiation of mesenchymal stem cells, regulation of mesenchymal cell function, fibroblast proliferation, and wound healing (Fig. 19.6) (Colwell et al. 2006; Jian et al. 2006; Salazar et al. 2009).

Wnt proteins promote the differentiation of fibroblasts into myofibroblasts, which express α-smooth muscle actin (α-SMA) (Akhmetshina et al. 2012). Myofibroblasts produce an extracellular matrix rich in collagen-1 and laminin and play a key role in fibrotic diseases. Cells of the inner sulcus of the cochlea demonstrate increased levels of α-SMA following EIT, which suggest an epithelial to mesenchymal cell transition (EMT) of these cells (Van De Water et al. unpublished data). These EMT cells may play an important role in the production of stress fibers and collagen type 1A in the lateral wall tissues and intracochlear fibrosis.

Bone morphogenic proteins (BMPs) are members of the TGF-β gene superfamily, which signal through Smad transcription factors. Interactions between BMPs and the Wnt/β-catenin pathway are crucial for osteoneogenesis (Fig. 19.6) (Chen et al. 2007; Chen and Alman 2009). Activation of these proteins may be responsible for new bone formation in the cochlea after CI-EIT.

In summary, CI-EIT can initiate an inflammatory process that leads to fibrosis and osteoneogenesis within the cochlea that can interfere with CI impedances and ease of CI reimplantation. The electrode array can also produce a foreign body reaction resulting in a chronic inflammatory condition that perpetuates scar and bone formation.

19.4 Drug Therapies to Prevent or Lessen Cochlear Implantation Trauma-Induced Sensory Cell and Hearing Losses

Corticosteroids and inhibitors of JNK signaling are two main drug classes that have been investigated for the treatment against CI-EIT-induced HC and hearing losses. The molecular mechanisms behind otoprotection by these two agents have been studied in depth and demonstrate consistent results among several studies.

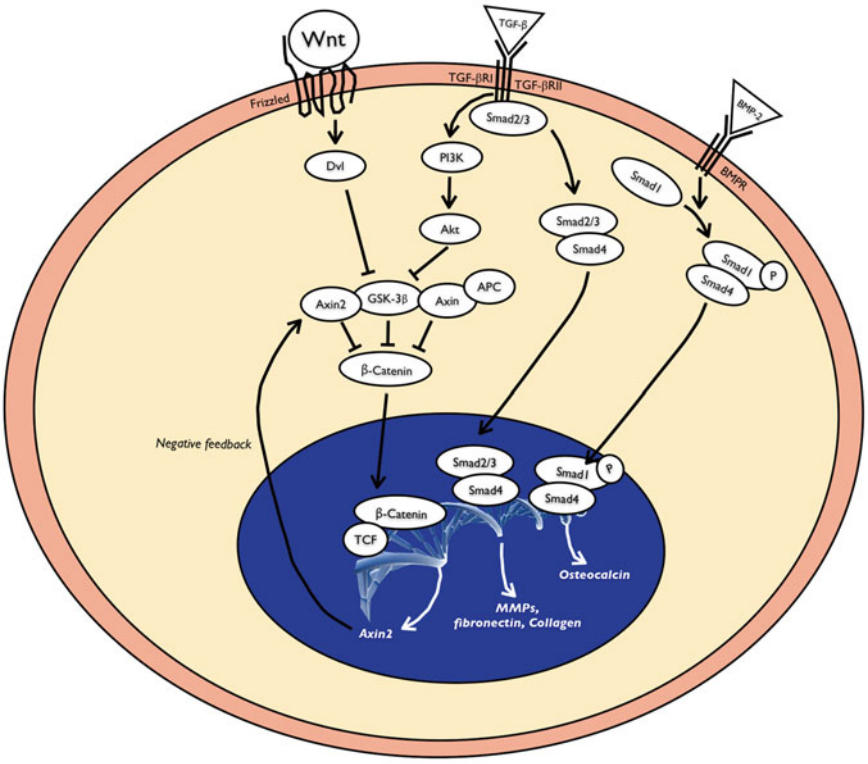

Fig. 19.6 TGF-β binds to its heteromeric receptor complex of type I and II and the intracellular signal is propagated downstream through Smads into the nucleus. Wnt signaling is initiated by secreted Wnt proteins, which bind to the Frizzled family of receptors. The activation of the receptor leads to the inhibition of GSK-3β-dependent phosphorylation of β-catenin and the stabilization and accumulation of cytosolic β-catenin. The accumulated β-catenin binds TCF/LEF transcription factors and activates transcription of target genes that will lead to the fibrotic tissue growth. TGF-β may cooperate with Wnt/β-catenin signaling. For example, TGF-β is known to activate PI3K which inhibits GSK-3β and could result in an increase of cytosolic β-catenin levels. BMP-2 binds its receptor and phosphorylates SMAD-1 and -5, which translocate into the nucleus and regulate the transcription of target genes, such as osteocalcin which is involved in bone formation

DXM is a potent synthetic corticosteroid that demonstrates strong anti-inflammatory and immunosuppressant effects. It is used routinely in the treatment of various inner ear disorders to reduce the inflammatory reactions that may promote death of auditory HCs and hearing loss following an insult to the cochlea. There are consistent results across several experimental studies in animals that have demonstrated the effectiveness of DXM treatment in preventing auditory HC death and hearing loss following an EIT event.

TNFα is a cytokine released during EIT and can activate the DR pathway of apoptosis and cross-communicate through tBid to activate mitochondrial death signaling; as a result, TNFα is used as an apoptotic stimulus to mimic an environment of EIT to test the effectiveness of DXM in protecting auditory HCs from EIT-induced loss.

DXM treatment of TNFα-injured OC explants in vitro demonstrated significantly higher levels of auditory HC survival when compared to explants damaged by TNFα without treatment (Dinh et al. 2008a). The result of several inhibitor studies conclude that DXM otoprotection is initiated when DXM traverses the cell membrane and binds to its glucocorticoid receptor (GCR) in the cytoplasm and activates pro-survival phosphatidylinositol 3-kinase (PI3K), Akt (also called protein kinase B, PKB), and nuclear factor kappa B (NFκB) proteins that lead to increased transcription of *Bcl-2* and *Bcl-xl* anti-apoptosis genes along with the downregulation of *Bax* and *TNFR1* gene expression levels resulting in an inhibition of caspase-dependent apoptosis (Haake et al. 2009; Dinh et al. 2008a). Similar mechanisms of action were demonstrated using polymer-eluted DXM against TNFα-initiated loss of auditory HC in vitro. DXM eluted from a biorelease polymer showed preservation of IHCs and OHCs that were comparable to levels of these sensory receptor cells present in control OC explants (Dinh et al. 2008b). Eluted DXM also mitigated expression of *Bax* and *TNFR1* genes associated with TNFα ototoxicity while also increasing expression of pro-survival *Bcl-2* and *Bcl-xl* in rat OC explants in vitro (Dinh et al. 2008b).

In an in vitro model of EIT, a 0.28 mm monofilament was inserted through a cochleostomy in an intact rat cochlea to simulate EIT. Subsequently, OC explants were dissected en bloc and cultured in vitro to study the molecular mechanisms associated with auditory HC loss following EIT and EIT explants treated with DXM. The expression levels of pro-inflammatory *TNFα* and *IL-1B* cytokines and *iNOS* and *COX-2* enzyme genes were significantly increased following EIT and these increases in gene expression levels observed in the EIT only explants were mitigated by DXM treatment of the EIT-OC explants. DXM treatment also blocked increases in the levels of ROS, cleaved caspase-3, and Endo-G that were demonstrated in EIT-OC explants (Bas et al. 2012). The culmination of these cellular events favored auditory HC survival following DXM treatment of EIT-OC explants. These results further support the anti-inflammatory and pro-survival modulatory effects of glucocorticoids following trauma-initiated auditory HC loss.

The effects of local DXM infusion into the cochlea, local DXM application to the round window niche, and DXM-eluting cochlear implant electrode arrays were tested against EIT in guinea pigs in vivo (Vivero et al. 2008; Eshraghi et al. 2007). EIT in guinea pigs elicited significant increases in ABR thresholds at 1, 4, and 16 kHz on post-EIT day 7 and permanent ABR threshold shifts at 0.5, 1, 4, and 16 kHz on post-EIT day 30 (Vivero et al. 2008; Eshraghi et al. 2007). Miniosmotic pump infusion of 100 μg/mL of aqueous DXM in artificial perilymph (AP) at a flow rate of 1 μL/h for 7 days reversed the temporary ABR threshold shifts seen in guinea pigs 1 week after electrode insertion (Eshraghi et al. 2007). The long-term effects of DXM on ABR threshold shifts were evaluated in guinea pigs that received miniosmotic pump infusion of 70 μg/mL of DXM base in AP at 1 μL/h for 8 days and these results demonstrated the reversal of EIT-induced ABR threshold shifts that were still present at post-EIT day 30 which represents a permanent conservation of hearing against EIT-induced loss (Vivero et al. 2008). The results of these studies collectively demonstrate that DXM can prevent EIT-induced auditory HC losses and permanent elevations in ABR threshold shifts.

DXM can be applied to the round window niche, diffuse across the round window membrane into the perilymph, and travel through the scala tympani from the basal turn to the apical turn of the cochlea (Plontke et al. 2008). In a guinea pig model of EIT, DXM was absorbed onto a carboxymethylcellulose/hyaluronic acid pledget and placed on the round window membrane for 30, 60, and 120 min prior to electrode array insertion. ABR thresholds were obtained 7 days post-surgery and levels of intracochlear DXM were measured. Higher levels of intracochlear DXM were achieved with higher concentrations of DXM (i.e., 20 % vs. 2 %) and with longer periods of exposure to the round window membrane (i.e., 120>60>30 min). ABR thresholds were significantly reduced at 1 week post-trauma for cochlea exposed to 2 % DXM for 60 min, 20 % DXM for 120 min, and 20 % DXM for 30 min at 16, 24, and 32 kHz; however, the best results of protection were demonstrated with 20 % DXM for 120 min (Chang et al. 2009). Similar protection of hearing against insertion trauma-induced hearing loss by DXM treatment was demonstrated when the middle turn of the cochlea was implanted instead of the basal turn (Eastwood et al. 2010a, b).

A single dose of intravenous DXM (2 mg/kg) administered 60 min prior to EIT in guinea pigs also demonstrated protection against permanent ABR threshold shifts 4 weeks post-trauma (Connolly et al. 2011). In another study, guinea pigs that received either one dose of systemic DXM (2 mg/kg) administered 60 min prior to EIT or local DXM (2 %) to the round window niche for 120 min prior to EIT demonstrated improved ABR threshold shifts that were still present at 3 months after the surgery/insertion trauma. There was no protection seen with 20 % DXM applied to the round window for 30 min and saline controls. Surprisingly, systemic DXM was associated with a significant reduction in the amount of fibrosis in the scala tympani 90 days following EIT compared to local DXM (2 %) for 120 min and saline control groups (Lee et al. 2013). A histological study of the cochleae of EIT guinea pigs after DXM treatment demonstrated lower levels of tissue reaction in their cochleae, fewer foreign body giant cells, decreased osteoneogenesis, and a reduction in the deleterious effects that result from an osseous spiral lamina injury (O'Leary et al. 2013). These histopathological findings were consistent with the ABR threshold results reported in this study.

The effects of SIBS-eluted DXM-coated electrode arrays were tested in guinea pigs in vivo that were subjected to insertional trauma from placement of these electrode arrays. Guinea pigs that received an uncoated or SIBS-coated electrode array inserted through a cochleostomy site into the scala tympani demonstrated significant ABR threshold shifts, particularly at 16 kHz that were permanent at 30 days post-implantation. However, guinea pigs implanted with electrodes coated with SIBS-eluted DXM arrays demonstrated a comparable short-term elevation of ABR thresholds that recovered to preoperative threshold levels by 30 days post-EIT. These findings were also confirmed with fluorescence labeling studies (i.e., FITC-phalloidin staining) demonstrating HC preservation with SIBS-eluted DXM electrodes (Eshraghi et al. 2011). These studies taken as an aggregate support the use of DXM administered either systemically or locally to protect residual hearing during and after the process of cochlear implantation, at least in animal models.

JNK inhibitors have also demonstrated otoprotection against EIT-induced loss of auditory HCs. EIT can initiate expression of MAPKs and in particular, a downstream members of the MAPK signaling cascade, c-Jun-N-terminal kinase (also referred to as JNKs, i.e., JNK-1, -2, and -3). Of these three isoforms of JNK (Zine and Van De Water 2004; Krishna and Narang 2008), two are expressed ubiquitously (i.e., JNK-1 and JNK-2) and the other is found primarily in the brain, heart, and testis (i.e., JNK-3). JNK is an enzyme capable of phosphorylating and activating the transcription factor c-Jun, which plays an important role in regulating the expression of several cell survival and death genes (Micheau and Tschopp 2003; Muppidi et al. 2004; Wang et al. 2003; Lei et al. 2002). D-JNKI-1 (D-JNK inhibitor peptide 1; also now known as AM-111) and SP600125 are two different drugs designed to target JNK, thereby inhibiting JNK molecules from activating transcription factor c-Jun and blocking cell death. D-JNKI-1 (renamed AM-111) has been shown to protect against EIT-induced hearing loss in guinea pigs (Eshraghi 2006; Eshraghi et al. 2013).

In a guinea pig model of EIT-induced hearing and HC losses, D-JNKI-1 was perfused into the cochlea using a mini-osmotic pump at 1 μL/h using 10 μM of D-JNKI-1 in perilymph for 1 week. Mean total changes of ABR threshold (at 0.5, 4, and 16 kHz) were calculated and were significantly reduced in ears treated with D-JNKI-1 compared to cochleae that received EIT alone, thereby demonstrating the protective effects of JNK inhibition in EIT 7 days post-trauma (Eshraghi 2006). In a similar study, prolonged effects of AM-111 were demonstrated up to 90 days post-EIT when AM-111 was suspended in a sodium hyaluronate gel at a concentration of 100 μM and pipetted onto the round window niche 30 min prior to electrode insertion. HCs, SG neurons, and HC-neuron synapses were preserved in the AM-111 treated cochleae exposed to EIT with this protection evident at 90 days post-EIT. Levels of phosphorylated c-Jun, ROS, and caspase-3 activation were significantly lower in auditory HCs of guinea pigs exposed to EIT and treated with the local application of AM-111 compared to EIT alone at 24 h post-trauma (Eshraghi et al. 2013). JNK inhibitors show great potential for protecting residual hearing following EIT that can occur during the process of cochlear implantation.

Two other agents that have been tested during cochlear implantation to improve hearing outcomes following CI surgery include L-*N*-acetylcysteine (L-NAC) and neurotrophin-3 (NT-3). L-NAC is a precursor to glutathione, which is an antioxidant important for neutralizing free radicals and ROS. The effect of L-NAC on EIT-initiated hearing loss in guinea pigs was tested by applying a pledget soaked in 40 mg/mL L-NAC on the round window membrane 30 min prior to insertion of an electrode into the cochlea. L-NAC abrogated ABR threshold shift at 24 and 32 kHz when compared to control untreated ears 4 weeks post-surgery. Although L-NAC demonstrated otoprotection at 4 weeks, there was a transient increase in hearing thresholds immediately after and at 1 week post-surgery that resolved by the end of the 4 weeks study. On histopathology, both control and L-NAC-treated ears demonstrated fibrosis around the implanted electrode array; however, fewer L-NAC-treated ears demonstrated a prolonged inflammatory reaction and expression of neutrophils, lymphocytes, and/or multinucleated giant cells. Surprisingly, more L-NAC-TREATED ears developed osteoneogenesis when compared to saline-treated control ears (Eastwood et al. 2010a, b). Although end results are promising

with respect to fibrosis, further studies are necessary to determine if L-NAC is a suitable drug overall for the protection of residual hearing against EIT-initiated loss given increased osteogenesis.

Another drug delivered to the cochlea is neurotrophin-3. Cochlear implant electrodes coated in electrically conducting polypyrrole/para-toluene sulfonate containing neurotrophin-3 (Ppy/pTS/NT-3) were inserted into the cochlea of guinea pigs deafened with aminoglycosides. Electrical stimulation of the implant resulted in release of 0.1 ng/day of neurotrophin-3 into the scala tympani compartment of the cochlea. Electrical stimulation occurred 8 h/day for 2 weeks. Greater SG neuron densities were observed with electrically stimulated Ppy/pTS/NT-3 electrodes compared to unstimulated Ppy/pTS/NT-3 electrodes and unimplanted cochleae (Richardson et al. 2009). These findings show great promise in hearing rehabilitation especially in patients with little to no baseline hearing prior to implantation.

In summary, EIT causes the release and expression of various pro-inflammatory and pro-death molecules that can activate DR (extrinsic) and mitochondrial (intrinsic) death pathways to promote caspase-dependent and -independent apoptosis of auditory HCs that lead to loss of residual hearing after cochlear implantation. Corticosteroids and JNK inhibitors have demonstrated short- and long-term protection against EIT-initiated HC loss and hearing loss by blocking several of these pro-death molecules, and results with antioxidants such as L-NAC have shown some promise as well.

19.5 Cochlear Implantation and Conservation of Residual Hearing

With advances in both electrode design and atraumatic surgical technique, low-frequency hearing preservation is possible following cochlear implantation with either conventional long electrodes (utilizing electric stimulation with the CI only for hearing), short electrodes, and hybrid electrodes [compatible with combined electroacoustic stimulation (EAS)]. Low-frequency hearing preservation following cochlear implantation is associated with improved postoperative hearing outcomes including: (1) pure tone thresholds, (2) speech perception in both quiet and noisy environments, and (3) melody recognition (Turner et al. 2004; Gantz et al. 2005, 2006, 2009; Gfeller et al. 2006; Büchner et al. 2009; Adunka et al. 2010, 2013; Carlson et al. 2011; D'Elia et al. 2012).

Despite the potential for immediate post-implantation low-frequency hearing preservation and improved hearing outcomes with soft surgical techniques and combined stimulation, several long-term studies demonstrate a progressive deterioration of residual hearing with time (Gantz et al. 2009; Santa Maria et al. 2013). This decrease in low-frequency residual hearing may be due to apoptosis of residual auditory HCs triggered by a chronic subclinical inflammatory process from electrical stimulation, a foreign body reaction to the CI itself, or maybe a progression of the patient's underlying disease process that produced the hearing loss in the first place. This decline in residual hearing long term will likely correlate with poorer hearing outcomes; however, further studies are necessary to confirm this theory.

Overall, the clinical benefits of the conservation of a patient's residual low-frequency hearing both during and after cochlear implantation are undisputed, and include improved pure tone thresholds, word recognition, understanding in noisy environments, and music appreciation. Recent studies that depict a decline in residual hearing long term may again turn the focus of electrode insertion design toward longer, less-traumatic electrodes that will compensate for future low-frequency residual hearing loss or drug-eluting electrodes that decrease the level and the duration of the cochlear inflammatory process and promote auditory HC and SG neuron survival over an extended period of time. By understanding the molecular signaling cascades that can occur in the cochlea during cochlear implantation, drug therapies can be designed and tested to target oxidative stress and key events that lead to intracochlear inflammation and to promote factors that lead to auditory sensory cell survival and improved CI benefits long term.

19.6 Conclusions

Conservation of a patient's residual low-frequency hearing is associated with improved hearing outcomes following cochlear implantation especially when combined EAS is utilized. Cochlear implantation trauma can initiate oxidative stress and the expression of various pro-inflammatory and pro-cell death signaling cascades that can lead to auditory HC and SG neuron losses and also intracochlear fibrosis. Soft surgical techniques and various drug therapies have been developed and tested to reduce the adverse molecular events that lead to loss of auditory HCs and deterioration of low-frequency residual hearing. Recent evidence suggests that residual hearing following cochlear implantation continues to decline even at years after the initial surgery despite immediate postoperative conservation of low-frequency audition. Future trends in cochlear implantation will likely involve the development of drug-eluting electrodes that can either slow or prevent this gradual deterioration of residual hearing. In addition, the development and implementation of longer, less-traumatic electrodes that can conserve residual hearing and that can compensate for loss of residual hearing are becoming a reality.

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Chapter 20
Preserving Residual Hearing in Cochlear Implant Patients

Thomas Lenarz and Verena Scheper

20.1 Cochlear Implants

The cochlear implant (CI) is recognized as best choice for severe/profound sensory hearing loss, for adults and children. It has been approved by Regulatory Authorities in most countries. Audible signals are detected by a microphone, processed into an electrical signal and transmitted transcutaneously to the implanted receiver. The signal is decoded and delivered through an electrode array implanted into the scala tympani of the cochlea (Fig. 20.1) onto the auditory nerve. The multichannel arrays have several contacts (12–22), depending on the brand and the product of different companies. The whole frequency range of the acoustic signal is split into different frequency bands and allocated to the different contacts, mimicking the physiological tonotopic organization of the cochlea. Biphasic charge-balanced electrical pulses delivered through these electrode contacts stimulate different parts of the auditory nerve eliciting different pitches (place coding). The time structure of the signal is coded by the rate of stimulation, the loudness via the amplitude of the stimulus.

Nearly 188,000 individuals worldwide are fitted with a cochlear implant (NIH 2014) which allows patients to understand speech in quiet environments and, more importantly, nowadays more and more recipients have speech understanding even in noise (Lenarz et al. 2013a). Nevertheless, CI users still have difficulty in identifying pitch, melody, and timbre efficiently (Kim et al. 2012) and novel technologies have to be developed to achieve satisfactory perception of sound, leading to appreciation and enjoyment of music.

Based on the excellent results achieved in profoundly deaf patients due to advances in implant technology and improved surgical techniques, also patients with severe

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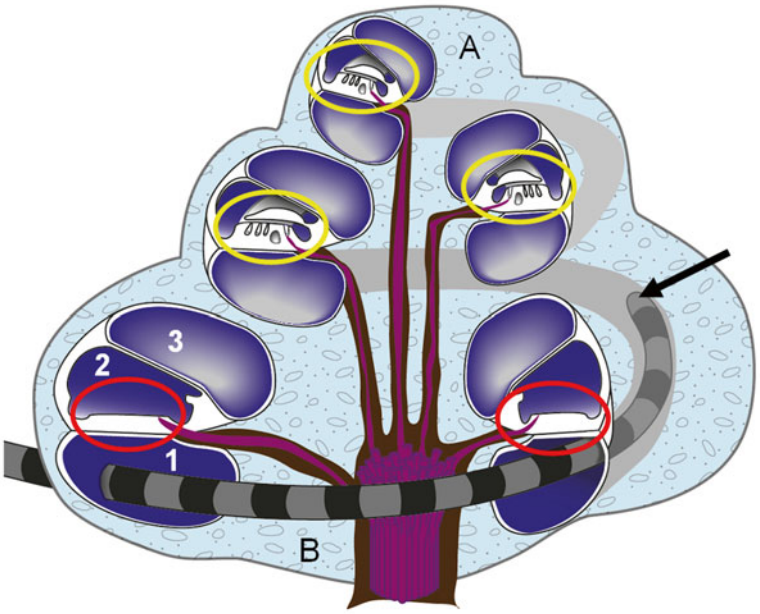

Fig. 20.1 Schematic drawing of a cochlea cross-cut. *A*: apical part, responsible for low-frequency hearing; *B*: basal part, responsible for high-frequency hearing; *1*: scala tympani; *2*: scala media; *3*: scala vestibule; *red circle*: Organ of Corti without hair cells, cochlear dead region; *yellow circle*: organ of corti with hair cells, residual hearing possible; *black arrow*: cochlea implant, short array to allow acoustic low-frequency hearing in the upper cochlear regions

hearing loss predominantly in the high frequencies and substantial residual hearing in the low frequencies are implanted with the goal of hearing preservation. Hearing aids do not provide the desired benefit in speech recognition in those patients (von Ilberg et al. 2011).

20.1.1 Electrical-Acoustical Stimulation

Continuing developments in implant electrode design and improved surgical techniques have resulted in increasing incidence of preservation of residual acoustic hearing in the implanted ear following cochlear implantation (Incerti et al. 2013; Lenarz et al. 2009), leading to expanded selection criteria in adult cochlear implant patients (Arnoldner and Lin 2013).

The apical portion of the basilar membrane within the cochlea is responsible for hearing in the low frequencies (Fig. 20.1A), while the basal turn is involved with audition in the high frequencies (Fig. 20.1B). Most patients suffer from hearing loss across all frequencies or from high-frequency hearing loss with residual hearing in low frequencies. If hearing in the apical region of the cochlea has been preserved in

cochlear implant patients, then the electrical stimulation of the implant is combined with a hearing aid in the same ear (electrical-acoustical stimulation, EAS) and patients can benefit from both: electrical stimulation from the CI and amplification of residual low-frequency hearing. This particular subset of patients who have residual low-frequency hearing can utilize their natural hearing to distinguish fine pitch differences that current cochlear implants cannot provide due to the limited number of separated channels (Bas et al. 2012a).

Recent data prove that EAS is a safe and effective method to provide patients with both benefits, the electrical stimulation in the deaf inner ear regions and acoustic hearing in the healthy parts (Gantz et al. 2009, 2010; Lenarz et al. 2013a; Mahmoud et al. 2014). Jurawitz et al. followed a large number of patients with different amounts of residual hearing using short and long atraumatic electrodes over a period of up to 2 years. Hearing thresholds were stable over time for the majority of patients with initial hearing preservation rates between 87 (longer electrode) and 93 % (shorter electrode) (Jurawitz et al. 2014). A study conducted by Mahmoud and colleagues followed up five EAS patients provided with the Med-El EAS system consisting of the $PULSAR_{CI}^{100}$ or $SONATA_{TI}^{100}$ implant, a $FLEX_{EAS}$ electrode array, and a DUET™ Speech Processor to support electrical cochlear stimulation and external hearing aid simulation. All participants showed preserved hearing in the surgical ear after implantation as measured by pure-tone audiograms and speech reception thresholds. Significant improvement in speech recognition testing over hearing aid was observed at 3 months with EAS vs. 6 months with CI-only stimulation. There were no significant complications in this cohort of patients. Adequate hearing preservation was achieved. EAS- and CI-aided conditions both showed significant improvement over hearing aid alone with better results in the EAS condition (Mahmoud et al. 2014). When comparing the percentage of correct answers to the HSM-speech test of patients provided with the FLEX system, it is obvious, that patients using the EAS instead of electrical stimulation only, perform better as shown by our own data (Fig. 20.2).

A multicenter study investigated the preservation of residual hearing in 66 subjects who received the Nucleus Hybrid L24 cochlear implant. The loss in mean air-conduction thresholds in the implanted ear for test frequencies 125–1,000 Hz was <15 dB across the population; both immediately and 1 year postoperatively. Sixty-five percent of subjects had significant gain in speech recognition in quiet, and 73 % in noise and combining residual hearing with CI gave a 22–26 % improvement in mean speech recognition scores over CI alone ($p \leq 0.01$). Useful residual hearing was conserved in 88 % of subjects and speech perception was significantly improved over preoperative hearing aids, as was sound quality and quality of life (Lenarz et al. 2013a). The EAS approach has already been proven to be beneficial in infants as well. A feasibility study was conducted to evaluate whether the use of a shorter-length cochlear implant (10 mm) on one ear and a standard electrode (24 mm) on the contralateral ear is a viable bilateral option for children ($n = 8$; age 12–24 month) with profound bilateral sensorineural hearing loss. A secondary purpose of the study was to determine whether the ear with the shorter-length electrode performs similarly to the standard-length electrode. Preliminary results for eight

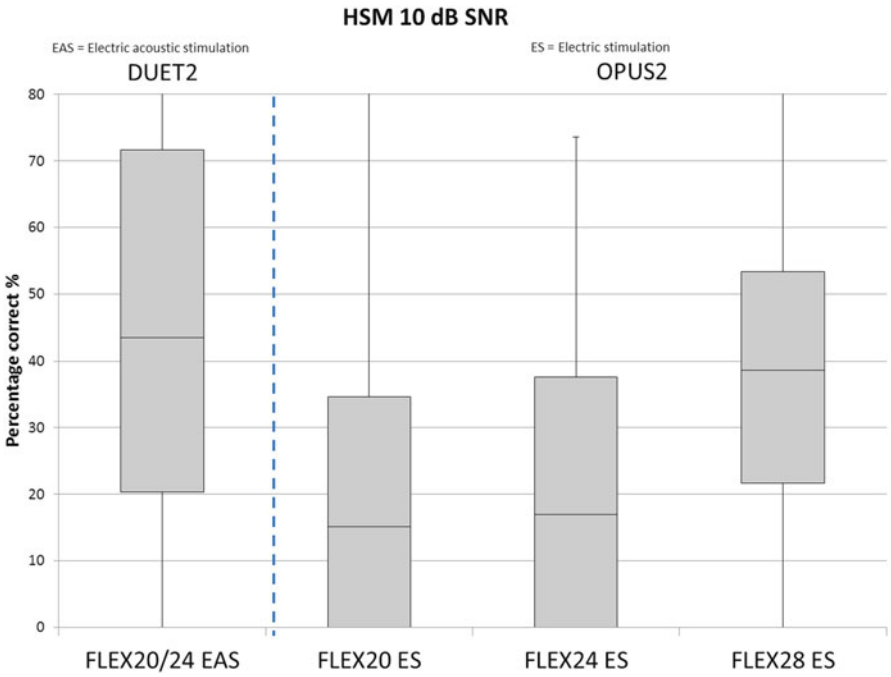

Fig. 20.2 Box-plot of the percentage of correct answers to HSM-speech test in patients using electric-acoustic-stimulation (EAS; *left box*) or electric stimulation only (ES; the three *right boxes*). Subjects were implanted with three different electrode length: 20 mm (FLEX20, $n=15$), 24 mm (FLEX24) or 28 mm (FLEX28). In EAS patients ($n=15$) a DUET2 speech processor was used whereas the ES patients were fitted with a OPUS2 processor (FLEX 20 ES: $n=22$; FLEX24 ES: $n=14$; FLEX28 ES: $n=27$)

children have been collected before and after the operation using the Infant-Toddler Meaningful Auditory Integration Scale (IT-MAIS). Three children showed incremental improvements in their IT-MAIS scores overtime. Early Speech Perception, Glendonald Auditory Screening Procedure word test, and Children's Vowel word perception results indicated no difference between the individual ears for the two children tested. Performance compared with age-matched children implanted with standard bilateral cochlear implants showed similar results to the children implanted with Nucleus Hybrid S12 10-mm electrode and a Nucleus Freedom implant in contralateral ears. The use of a shorter-length cochlear implant on one ear and a standard-length electrode on the contralateral ear might provide a viable option for bilateral cochlear implantation in children with bilateral profound sensorineural hearing loss, the authors concluded (Gantz et al. 2010).

To allow the comparison of results of different clinical trials investigating residual hearing preservation in CI patients a group of experts introduced a Hearing Preservation Classification System, fulfilling the following necessary criteria: (1) classification is independent from users' initial hearing; (2) it is appropriate for all cochlear implant users with measurable preoperative residual hearing; (3) it covers

the whole range of pure-tone average from 0 to 120 dB; (4) it is easy to use and easy to understand (Skarzynski et al. 2013).

Unfortunately, post-implantation hearing loss occurs in a certain percentage of patients. Besides insertion depth, length, and mechanical characteristics of the electrode in use and the surgical technique other factors might contribute. The insertion of the electrode array into the inner ear during cochlear implantation can lead to foreign body and trauma reaction and a deterioration of the residual hearing. The postoperative result is influenced by five main factors besides multiple other biological mechanisms:

(a) Electrode characteristics and surgical technique
(b) Insertion trauma
(c) Foreign body reaction
(d) Electrode–neuron-interfacing
(e) Long-term stability of electrode position and function

20.1.2 Electrode Characteristics and Surgical Technique

The design of the electrode array and the choice of the surgical technique are two highly relevant factors influencing the residual hearing in cochlear implant patients. Since electrode characteristics like stiffness are well known to have a high impact on preservation of residual hearing (Tamir et al. 2012), the insertion depth of the electrode array is still controversially discussed. In some studies implantation with a standard electrode array resulted in a significant risk of destroying residual hearing in the low frequencies (Ching et al. 1998; Vickers et al. 2001). And in a histological study using fresh human temporal bone specimens, smooth electrode insertions through a cochleostomy resulted in shallower insertion depths but associated significantly less intracochlear trauma compared with more forceful and deep insertions (Adunka and Kiefer 2006). Next to this, other studies did not report an effect of electrode insertion depth on residual hearing if relatively long electrode arrays of low stiffness are used (Tamir et al. 2012). While previous papers show hearing preservation rates that range from 50 to 81 % (Baumgartner et al. 2007; Skarzynski et al. 2002), the latest papers demonstrate hearing preservation rates ranging from 77 to 100 % in a large number of child and adult subjects (for review see Bas et al. 2012a). Jurawitz et al. could demonstrate on a large patient population that electrode length and insertion depth are relevant for hearing preservation with a higher percentage of preservation for a short electrode (16 mm) compared to a longer electrode (20 mm insertion depth) (Jurawitz et al. 2014).

This enormous increase of residual low-frequency hearing preservation is due to a novel generation of cochlear implants with atraumatic, often shorter electrode arrays, avoiding too deep insertion in the relevant patient population. The available cochlear implants with shortened electrode array are the “Hybrid-system” from Cochlear LTD (Sydney, Australia) with an electrode length of 10 mm (“Hybrid-S”), 15 mm (“Hybrid-M”) and 24 mm (“Hybrid-L”) and the “Flex-EAS-System” from

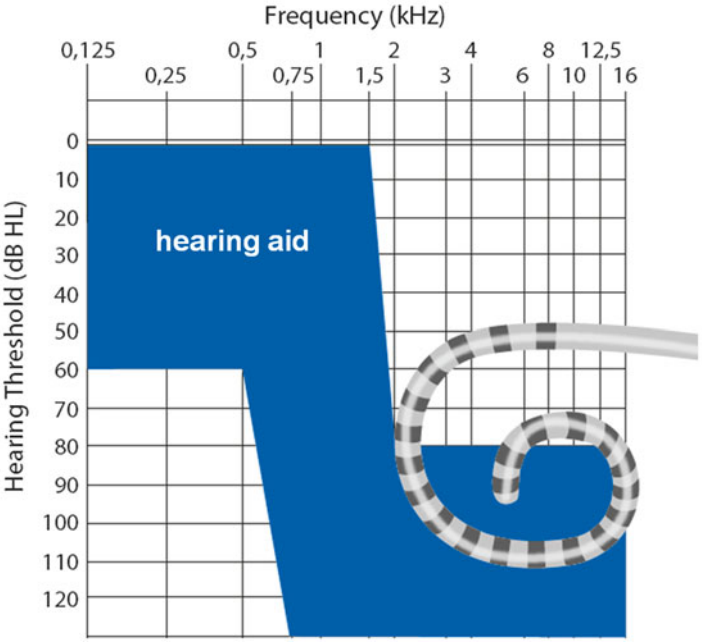

Fig. 20.3 This figure illustrates the indication range for short electrode arrays: the target ear needs to fall within the shaded region

MEDEL (Innsbruck, Austria) with an electrode length of 20 mm ("Flex 20"), 24 mm ("Flex 24") and 28 mm ("Flex 28"). The indications for hearing aid and cochlear implant therapy are visualized in Fig. 20.3.

There are different methods the surgeon has on hands to choose the right electrode length in each individual case of residual low-frequency hearing. On the one hand one should base the decision on the tone audiograms. As better the hearing in lower frequencies as shorter the electrode array should be. The second, and more precise, method to use as basis for the decision of electrode array length is imaging of the cochlea before surgery since the individual cochlear anatomy dictates the maximum electrode array insertion depth. Digital volume tomography (DVT) or high-resolution computer tomography (HRCT) are methods routinely used preoperatively in cochlear implant patients for diagnosis of e.g., hydrops or ossifications relevant for planning the surgery. Those datasets can be used to measure the length of the cochlea, resulting in an exactly individualized prediction of which electrode array length may fit into a specific cochlea (Würfel et al. 2014). Basic measurements of the cochlear dimensions, including cochlear axis height, width of the cochlear, base, and length of the cochlear base, show variability of cochlear parameters ranging from 12 to 19 % of the maximum value, proving a tremendous variability in cochlea anatomy (Avci et al. 2014). A significant positive correlation between length of the cochlear base and width of the cochlear base as well as a variability of the number of cochlear turns was variable, the mean being 2.64 ± 0.17 ($949 \pm 62°$), ranging from

2.39 to 2.84 (859–1,024°) (Avci et al. 2014). Pelliccia and colleagues were able to prove that there is no age-relation in cochlea size, since the cochlea has already reached adult size at birth. They evaluated the cochlea length and height, basal turn lumen diameter and volume of the cochlea and concluded that the degree of deafness does not affect the length or volume of the cochlea, while it can affect height and basal turn lumen diameter (Pelliccia et al. 2014). In order to preserve residual hearing they propose a straight electrode with three landmarks on the array (the first at 16.635 mm from the tip, the second at 17.987 mm, the third at 19.34 mm) to assist the surgeon in obtaining the ideal insertion depth angle of 270° when a preoperative measurement of cochlear length by high-resolution computer tomography has been performed (Pelliccia et al. 2014).

The electrode array length and stiffness are not the only cochlear implant characteristics influencing the outcome of the implantation on residual hearing. Next to this, the positioning of the array in the scala tympani, due to precurving of the array, and the arrays diameter are relevant factors.

Conventional *straight electrodes* and *contoured electrodes* are preserving residual hearing using a cochleostomy approach (Berrettini et al. 2008; Fraysse et al. 2006; Garcia-Ibanez et al. 2009) and there are published results on the hearing preservation outcome demonstrating that there is no significant difference in hearing preservation between these non atraumatic straight and perimodiolar electrodes. Soda-Merhy and colleagues compared straight Nucleus 24 K (Cochlear Corp, Australia), MEDEL Combi 40+ (MEDEL, Innsbruck, Austria) and HiRes 90 Focus 1j (Advanced Bionics, Valencia, CA) and the perimodiolar Nucleus 24 Contour and Nucleus 24 Contour Advance (both Cochlear Corp, Australia) in a clinical trial with 48 patients using a cochleostomy approach. They reported no statistically significant difference in the preservation of residual hearing between straight or contour arrays with similar rates for both electrode designs across frequencies (Soda-Merhy et al. 2008).

When inserting the Contour Advance electrode through the round window in human temporal bones ($n = 16$) successful full insertions of the electrode with minimal resistance and good perimodiolar placement was infrequently achieved (Souter et al. 2011). A common finding was obstruction of electrode entry at the anteroinferior margin of the round window, which prevented optimal scala tympani positioning and often resulted in intracochlear trauma. The anteroinferior region of the round window bony margin influences the trajectory of insertion of the relatively large Contour Advance electrode as well as physically obstructing and distorting the array. A pure round window insertion is not predictable and reliable enough to be a recommended approach for the used electrode (Souter et al. 2011). This leads to the conclusion that the surgical technique is also influencing the outcome of cochlear implantation. While the surgical approach—*round window vs. cochleostomy*—seems to be highly important for the preservation of residual hearing using contoured arrays, the choice of the array insertion-point seems less important when using atraumatic straight electrode arrays. Straight electrodes have been used with round window and cochleostomy insertions with good results for protecting residual auditory function in the low frequencies (Havenith et al. 2013; Lenarz et al. 2013a;

Skarzynski et al. 2011; Soda-Merhy et al. 2008). Studies comparing cochleostomy and round window insertion show that hearing preservation cochlear implantation can be performed via a cochleostomy or via the round window membrane itself with similar outcomes in terms of both hearing preservation rates as well as speech perception measures (Adunka et al. 2014; Nguyen et al. 2013). However, when comparing the results of two multicenter studies using the Hybrid-L electrode, better results were documented with the round-window approach (Lenarz et al. 2013a vs. US trial reported by Zhou et al. 2014). The round-window approach avoids intensive drilling on the cochlea, the formation and incorporation of bone dust, and accidental suctioning while removing bone dust.

The *tip diameter* is an essential criterion for the array design to improve hearing preservation results. When comparing pure-tone audiograms of patients implanted with various surgical techniques and three array designs, 3 months postoperatively, hearing preservation within 30 dB was achieved in 50 %, 50 %, and 84 % cases of patients implanted with a Contour Advance, Flex-EAS, and Hybrid-L, respectively. Best results were achieved using arrays with small-tip diameters (Nguyen et al. 2013).

To reduce the surgically induced trauma the so-called "soft surgery techniques" were developed. Lehnhardt was the first to describe a procedure for CI insertion to preserve residual low-frequency hearing (Lehnhardt 1993). Over the years improvements of Lehnhardts surgery method were developed to increase the protection of residual hearing ability (Backous 2014; Cohen 1997; Kiefer et al. 2004; Lenarz et al. 2009; Postelmans et al. 2011; Roberson 2005; Roland et al. 2005).

Parameters of the soft surgery-technique are a slow electrode insertion with the least pressure and coverage of the insertion site of the electrode array using muscle or fascia.

The *insertion speed* has a significant impact on various insertion characteristics as well as hearing preservation and vestibular function. Force measurements performed while inserting human electrodes in an artificial scala tympani model at progressive increase in insertion speed from 10 to 200 mm/min resulted in significant, proportional increase in the average insertion forces from 0.09 to 0.185 N and in the maximum forces from 0.18 to 0.42 N (Kontorinis et al. 2011). Additionally, the insertion speed was measured through videos of 116 human implantations showing an average insertion speed used in the surgical theaters during human cochlear implantations of 96.5 mm/min (range, 42–165.2 mm/min) and depended on the electrode type and the surgeon (Kontorinis et al. 2011). Other clinical data obtained during hearing-preserving cochlear implantation using systemic and topical steroids in conjunction with a round-window approach, a complete cochlear coverage electrode and two different electrode insertion speeds [60 mm/min ($n=18$) vs. 15 mm/min ($n=22$)] proved that a slow electrode insertion speed appears to facilitate full electrode insertion, reduce the occurrence of insertion resistance as well as promote preservation of residual hearing and vestibular function after cochlear implantation (Rajan et al. 2013).

Several different *sealing techniques* are used in human or animal studies to seal the insertion site to prevent leakage of perilymph or ingress of air and tissue formation, including no seal (Skarzynski et al. 2007) as well as an autologous muscle or

fascia graft (Braun et al. 2011; Lenarz et al. 2013a; Prentiss et al. 2010). A recently published study using an animal model compared the effect of different sealing techniques—no seal, muscle graft or carboxylate cement—in relation to the surgical approach—round window and cochleostomy—on residual hearing performance after cochlea implantation. Almost no difference with regard to hearing thresholds was observed between the no seal and muscle seal groups. In all animals using the cement an almost total loss of residual hearing was observed. Therefore the use of carboxylate cement as a sealing material in cochlear implantation should be avoided even in animal studies, whereas sealing the insertion site with a muscle graft does not induce an additional tissue growth compared to omitting a seal (Burghard et al. 2014).

20.1.3 Insertion Trauma

Both, electrode characteristics and surgical technique, may lead to mechanical injury of the basilar membrane or osseous spiral lamina, which can affect the endocochlear potential, create oxidative stress, and initiate proapoptotic pathways associated with direct injury to and loss of HCs (Bas et al. 2012a). At the molecular level, insertion of a cochlear implant electrode array causes direct tissue trauma and cell losses via necrosis, but it also generates molecular events that will contribute further to a loss of any residual hearing: for example, oxidative stresses and release of proinflammatory cytokines that can lead to the initiation of programmed cell death (Eshraghi et al. 2013). Even though to our knowledge the exact mechanisms involved during cochlear implant surgery that results in loss of residual hearing is not completely understood recent findings on caspase activation, JNK activation, oxidative stress with ROS, and lipid peroxidation of cellular membranes (Eshraghi et al. 2013) give a first hint on possible targets for drug-based therapies of insertion trauma-related hearing loss. (See Chap. 19 by Bas et al. for a detailed discussion of cell death pathways associated with cochlear implantation.)

Studies on *local drug delivery* to the inner ear to prevent loss of residual hearing are mainly focussed on corticosteroids, JNK inhibitors, and antioxidants. It is believed that corticosteroids such as *dexamethasone* (DEX) can protect against death of HCs through inhibition of proinflammatory and proapoptotic triggers that initiate intrinsic and extrinsic cell death and prevents recruitment of additional macrophages to the site of injury and adjacent sites (Bas et al. 2012b; Tizard 2004). Using an animal model it was possible to attenuate progressive hearing loss associated with electrode implantation trauma when dexamethasone was applied locally into the inner ear via a miniosmotic pump (Vivero et al. 2008). Additionally in vivo studies indicate a decrease in the early elevated hearing thresholds observed following cochlear implant surgery to preoperative levels 1 month following initiation of DEX treatment (Eshraghi et al. 2011) and recovery of the CAP or ABR threshold following corticosteroid treatment in implanted inner ears (Braun et al. 2011; Eastwood et al. 2010a; Ye et al. 2007).

C-Jun N-terminal kinases (*JNKs*), a class of mitogen-activated protein kinases, are suggested to be involved in insertion-trauma-induced hearing loss since inhibitors of JNK-signaling have been demonstrated to protect HCs from degeneration and residual low-frequency hearing from decline after electrode insertion trauma. DJNK inhibitor-1 (DJNKI-1, also known as AM-111) treatment substantially attenuated ABR threshold shifts and changes in distortion product otoacoustic emissions (DPOAE) associated with immediate and progressive auditory dysfunction in an animal model of electrode insertion trauma (Eshraghi et al. 2007).

Lastly, there is an increasing role *for antioxidants* in the protection of residual hearing loss as there is increasing evidence linking high levels of oxidative stress to programmed cell death of remaining HCs. Knowledge of the mechanisms underlying noise- and drug-induced hearing loss (NIHL, DIHL) has led to the hypothesis that cochlear-implantation-related hearing loss is based on the same pathophysiology (see also Chap. 19 by Bas et al.). Noise, ototoxic drugs, and other stress agents induce free radical formation in the inner ear, which upregulate apoptotic cell death genes. If free radical formation is sufficient, cell membranes are attacked, resulting in necrotic cell death. Aerobic organisms require molecular oxygen (O_2) for vital cellular processes. As the consequence of respiration and enzymatic activities, cells can generate partially reduced forms of O_2 collectively referred to as "reactive oxygen species" (ROS). The gaseous molecule nitric oxide (NO) and its derivatives, also produced intracellularly, define a subclass of ROS termed reactive nitrogen species (RNS) (Ryter et al. 2007). The production of ROS/RNS in excess of an endogenous cellular capacity for their detoxification and/or utilization results in nonhomeostatic states referred to as "oxidative" or "nitrosative" stress, respectively. In addition to metabolic production, which is governed in part by O_2-tension, a multiplicity of xenobiotics, drugs, cytokines, and environmental factors (i.e., solar ultraviolet radiation, ionizing radiation, and cigarette smoke) can elevate intracellular ROS production (Ryter et al. 2007). ROS can cause the progressive modification or degradation of cellular biochemicals, including DNA, protein, lipids, and carbohydrates, when produced at elevated nonphysiological concentrations. Such cumulative damage induced by ROS can lead to loss of cell function or cell death. Consequently, ROS have been implicated in the aging process, in tumorigenesis/carcinogenesis, and in the progression of various pathologies (Essick and Sam 2010), such as cardiovascular diseases, neurodegenerative disorders, and rheumatoid arthritis. In addition to their roles in subcellular damage, an emerging hypothesis argues that ROS exert physiological effects or functions in the signaling pathways that regulate cellular processes, including gene expression, growth, and regulated forms of cell death (e.g., apoptosis) (Herrera et al. 2001).

Exogenous antioxidants such as sodium thiosulfate (STS, Neuwelt et al. 1998); D-methionine (D-met, Samson et al. 2008); ascorbic acid (vitamin C, Derekoy et al. 2004); tocopherol (vitamin E, Hou et al. 2003); and *N*-acetylcysteine (NAC, Eastwood et al. 2010b; Lu et al. 2014) (Kannan and Jain 2000) have shown some degree of benefit against cisplatin, carboplatin, aminoglycoside, and noise-induced trauma to the inner ear in various studies (Dinh and Van De Water 2009). However, none of these studies address hearing loss associated with electrode insertion trauma

and cochlear implantation except for Eastwood et al. who were able to demonstrate benefit with NAC treatment (Eastwood et al. 2010b). This benefit only pertained to the high frequencies of the basal turn and did not extend significantly to low-frequency hearing located in the apical section of the cochlea. Unfortunately, delivery of NAC to the round window prior to implantation caused a slight increase in hearing thresholds and greater amounts of osteoneogenesis, which may preclude its use locally in the protection of residual hearing (Eastwood et al. 2010b). No study was performed on evaluation of antioxidant effects on cochlear implant electrode insertion-related hearing loss.

In 2013 a single-side, randomized clinical trial started in the Department of Otolaryngology at Hannover Medical School (MHH), Germany. In this double-blind, placebo-controlled phase II study for the first time the potential hearing preservation in cochlear implant patients with residual hearing mediated by a chewable tablet with antioxidative and vasodilative effects is investigated (www.pro-hearing.eu). The tablet components, the vitamins A, C, E, and magnesium (ACEMg) belong to the pharmacological class of vitamins and micronutrients. ACEMg is a formulation composed of a micronutrient combination of 500 mg vitamin C (magnesium ascorbate), 315 mg magnesium (magnesium citrate, magnesium ascorbate, magnesium stearate), 267 mg vitamin E (α-tocopherol acetate), and 18 mg beta carotene.

The strategy has already been evaluated in a series of international human clinical trials treating temporary (military gunfire, audio player use) and permanent (stamping factory, military airbase) hearing threshold shift models (NCT00808470) to investigate the effect of ACEMg during noise exposure.

20.1.4 Foreign Body Reaction

After CI surgery the acuity of residual hearing and that of CI-mediated hearing are often affected by postoperative intracochlear fibroblast growth. A histological evaluation of human temporal bones from CI patients revealed inflammatory cell infiltrates around the implant in 57 % of the examined cases, likely due to a sterile inflammation produced by mechanical trauma during surgery as well as a persistent foreign body response of the host tissue to the implant (Nadol and Eddington 2004). These findings were supported by an additional human temporal bone study which defined further the involved cells (Nadol et al. 2008).

Damage to spiral ligament, Organ of Corti, osseous spiral lamina, and other fine structures in the inner ear may possibly be reduced by optimization of CI design, but still the occurrence of inflammation, either acute or chronic, cannot be prevented (Nadol and Eddington 2004). As a consequence, increased impedance, reduced speech perception, and functional derogation of the device itself may take place. Two strategies, as single approach or in combination, are investigated to functionalize the electrode array surface with the aim of reduction of fibrous tissue growth and related reduction of residual hearing loss. *Physical* changes of the array such as specific surface patterns with structures in the micro- and nanometre range can be used to

manipulate cell morphology, proliferation, adhesion, migration, and differentiation in vitro (Curtis and Wilkinson 1998; Ito 1999; Meredith et al. 2007). For platinum as a contact material used in cochlear implants it was shown that linear grooves of 4–7 μm in width are able to reduce fibroblast growth in vitro compared with unstructured controls (Reich et al. 2008). Nanostructures can influence the cell-surface interaction by modifying the surface properties such as wettability, light absorption, or water contact angle (for review see Lenarz et al. 2013b). *Chemical* functionalization is based on coating of the implants surface. An overview of possible types of permanent and degradable coatings is given in Sousa et al. (2003). Typically, polymer coatings are used: either passive ones or active ones that carry bioactive substances and act as a drug delivery system. For preservation of residual hearing mediated by manipulation of foreign body reaction, mainly release of dexamethasone has been studied. It was shown that dexamethasone, incorporated in the base material of the implant [polydimethylsiloxane (PDMS)] which was than coated with a hydrogel layer [star-shaped polyethylene glycol prepolymer (sPEG)] to prevent cell and protein adhesion, reduced fibroblast growth on the surface and that the hydrogel coating reduced and prolonged the release of the drug over several months (Wrzeszcz et al. 2013). In another study dexamethasone was eluted from poly(styrene-b-isobutyl-eneb-styrene) to protect hair cells against ototoxicity of tumor necrosis factor α (Dinh et al. 2008), supporting the application of dexamethasone containing polymer onto electrode arrays for the conservation of hearing during cochlear implantation. Paasche et al. (2006) conducted a clinical study in adult Cochlear implant recipients comparing the effects of microstructured electrode surfaces with iridium deposits and intracochlear injection of triamcinolon crystal suspension. The usual increase of electrode impedances was significantly reduced with triamcinolon. These results were interpreted as a reduction of fibroblast growth around the electrode through the action of the steroid (Paasche et al. 2006).

20.1.5 Electrode–Neuron-Interfacing

One major cause for deafness is hair cell loss followed by retraction of the peripheral processes of the spiral ganglion neurons (cell bodies of the hearing nerves) and, after longer lasting untreated deafness, degeneration of the spiral ganglion neurons themselves. This retraction of the peripheral neurons causes a large distance between the implant device which is positioned in the region of the formally Organ of Corti and the hearing nerve. Decreasing this distance would advance the channel discrimination, increase the frequency-specific hearing, and improve the energy consumption of the implant. The need for neuronal attraction, guidance, and regrowth towards an interfaceable device is important in all medical fields, from spinal cord injury and disorders of sensory systems therapy such as retinal and cochlear implants towards diseases of the central nervous system like Parkinson disease and neuroblastoma. Research in this field related to cochlear implants has just started and first studies indicate that it is possible to increase neurite outgrowth or even attract spiral ganglion neurites out of the bony cochlear modiolus into the scala tympani where the cochlear

implant is inserted. In all cases, neurotrophic factors such as brain-derived neurotrophic factor (BDNF, Leake et al. 2011; Shibata et al. 2010) or BDNF in combination with neurotrophin-3 (NT-3) (Landry et al. 2013; Staecker et al. 1996; Wise et al. 2005, 2010) or acidic fibroblast growth factor (aFGF) (Glueckert et al. 2008) as well as factor cocktails (Wise et al. 2011) have been used. Occasionally SGN neurites projecting across the osseous spiral lamina into the scala tympani are observed (Glueckert et al. 2008; Landry et al. 2013; Leake et al. 2011; Staecker et al. 1996; Wise et al. 2011). Many of these neurites projecting into the scala tympani, as well as a small number that projected onto the basilar membrane, had a swelling at the tip reminiscent of a growth cone, although this could also be caused by an abnormality in the structural protein matrix of the neurites (Landry et al. 2013). There would be a number of functional advantages if the growth of neurites into the ST could be routinely promoted and directed toward the proximal electrode. Although there have been some attempts to develop stimulating electrodes that release neural guidance cues such as neurotrophis to attract neurites, this work is in its infancy (Hütten et al. 2013; Scheper et al. 2009; Thompson et al. 2010; Warnecke et al. 2007, 2010).

20.1.6 Long-Term Stability

Long-term stability of residual hearing can only be guaranteed if all events typically following all surgeries, such as infections and inflammations, are eliminated or at least very significantly diminished.

Postoperative bacterial infections, *biofilm formation* of the CI surfaces, and especially ascending infections via the Eustachian tube in the middle ear pose prominent problems in CI implantation that currently are not being handled effectively (Kanaan et al. 2013; Kos et al. 2009; Makarem et al. 2008). At present, the prevention of CI infections is carried out by perioperative systemic antibiotic treatment and standard vaccination against *Streptococcus pneumoniae* and *Haemophilus influenzae* (Antonelli et al. 2004; Makarem et al. 2008). However, the parenteral administration of antibiotics involves drawbacks such as systemic toxicity and prolonged hospitalization to monitor drug levels and its effects. In order to avoid these drawbacks, local antibacterial administration has become an accepted alternative to systemic antibiotic therapies in some fields. These therapies offer various advantages, including a high drug concentration at the potential infection site while eliminating systemic toxicity, a more complete eradication of the local infection and the use of smaller drug doses. Drug-loaded biomaterials with non-fouling properties have been proposed for local drug administration. Such materials are capable of releasing drugs immediately after the surgery and at least during the following 6–72 h (Yeap et al. 2006; Zalavras and Patzakis 2003). This covers the critical period of possible bacteria attack and proliferation in the intervention site.

Following cochlear implantation, fibrous tissue is formed around the electrode array due to *inflammation*. The amount of tissue formation can vary between little loose fibrous tissue and new bone formation (Somdas et al. 2007) and is thought to cause the increase in electrode impedance (Tykocinski et al. 2005) as measured

during the first 2–3 weeks after implantation. Currently, several strategies such as coating-mediated drug delivery (Dinh et al. 2008), drug delivery from the electrodes basic silicone material (Jolly et al. 2010) or surface patterning (Fadeeva et al. 2013) for reduction of fibrous tissue growth around the electrode array are under investigation.

For both insults, inflammation and infection, local drug delivery on demand is the future perspective. Worldwide research is ongoing to investigate an implant-based system which allows local drug delivery in the cochlear implant patient not routinely but in situations where it could be beneficial for the implants healing and preservation of residual hearing. Strategies may include temperature or pH-changes, electroporation for gene delivery (Pinyon et al. 2014), ultrasound (Huebsch et al. 2014), coatings for sustained release (Ceschi et al. 2014; Chikar et al. 2012) and pump-based delivery (Johnson et al. 2010).

Next to the inflammatory processes, which are believed to be one of the reasons for increased impedances after cochlear implantation, SEM micrographs of platinum-contact cross sections of an explanted CI with formally high impedance values revealed surface irregularities on the affected electrodes. Surface erosion could either be caused by external factors such as a local inflammation process, or result from dissolution of the platinum itself (Durisin et al. 2011). In vitro experiments on platinum dissolution demonstrated different dissolution rates depending on the charge density in anodic first pulses and in cathodic first pulses primarily on pulse duration (McHardy et al. 1980). Whether local cochlear inflammation cause increases in impedance by formation of connective tissue or even bone along the electrode and/or production of aggressive inflammatory exudates, which corrode the electrode surface, these factors remain to be elucidated in future carefully performed studies. Additionally, we need to determine if the electrical stimulation parameters used may lead in some special situations to an electrotoxicity, where the electrodes' platinum is affected and destroyed by the electrical stimulation itself and damage of hair cells or other cochlear cells are due to overstimulation exceeding charge density safety limits for these cells (Neuburger et al. 2009). In that case optimized and safe stimulation strategies e.g., changes in stimulation rate and range, pulse duration as well as the setting of C and M levels have to be established.

20.2 Summary and Future Directions

Cochlear implants are the success story of neuroprosthetic devices with a fast growing number of recipients worldwide. Recent advances in implant technology provide open set speech understanding to the majority of patients. However, current limitations exist for speech in noise understanding and music listening. Two major factors at the electrode–nerve interface are contributing:

- The limited number of separated electrode channels for information transmission on the technology site
- The remaining number of auditory neurons resisting degeneration and the degeneration of dendrites which prevents the direct electrode–nerve contact

Both limits can be pushed towards better solutions addressing all five relevant parameters.

1. Electrode mechanics can be improved by individualized implants which allow an optimized fit to the patient's cochlear anatomy.

   The surgery will become more precise and less traumatic to preserve both residual hearing and neurons. High resolution cone beam CT data can be used to create a model of the cochlea which will allow a proper selection of the electrode and definition of the optimum insertion pathway. The precise insertion itself will be robot assisted.
2. The trauma reaction can be reduced by controlled local drug delivery systems incorporated into the electrode array with multiphasic kinetics for initial and sustained release of corticosteroids and other relevant drugs. Antioxidants should be released locally to guarantee an effective concentration.
3. Foreign body reaction can be markedly reduced or even avoided by functionalization of the electrode surface through nanostructuring and polymer coating.
4. The electrode–nerve interface will be markedly improved by formation of dendrite regrowth onto the functionalized electrode surface. This will allow the design of electrodes with much smaller contact areas and significant increase in the number of separated channels.
5. The long-term stability of the electrode can be improved using local drug delivery systems with an on demand release mechanism of antiinfective and antiinflammatory drugs. Constant monitoring of electrode impedances will allow for early detection of changes of electrophysiological patterns to adapt the electrical stimulation parameters and avoid electro- and neurotoxicity.

All these measures will contribute to a constant improvement in performance and reduce complications.

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Chapter 21
The Role of Oxidative Stress in Idiopathic Sudden Sensorineural Hearing Loss and Meniere's Disease

Wong-Kein Christopher Low, Russel Kahmke, and Debara L. Tucci

Abbreviations

AMPA	Alpha-amino-3-hydroxyl-5-methyl-4-isoxazole-proprionic acid
DMSO	Dimethylsulfoxide
ELH	Endolymphatic hydrops
GSH	Glutathione
ISSNHL	Idiopathic sudden sensorineural hearing loss
JNK	C-Jun *N*-terminal kinase
LNAC	L-*N*-acetylcysteine
MD	Meniere's disease
NADPH oxidase	Nicotinamide adenine dinucleotide phosphate-oxidase
NKCCl	Na–K–2Cl cotransporter
NMDA	*N*-methyl-D-aspartate
NO	Nitric oxide
NOS	Nitric oxide synthase
OHC	Outer hair cells
RA	Radix astragali
ROS	Reactive oxygen species

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21.1 Idiopathic Sudden Sensorineural Hearing Loss

21.1.1 Introduction

Sudden sensorineural hearing loss is most commonly defined as a loss of at least 30 dB in three contiguous frequencies over a time course of 72 h or less (Stachler et al. 2012). A cause can be identified in only about 10 % of patients, including bacterial infections, circulatory disorders, traumatic injuries, immunologic, toxic, neoplastic, metabolic, and neurologic sources. Popular causative theories of idiopathic sudden sensorineural hearing loss (ISSNHL) point to viral infection, cochlear membranous breaks, autoimmunity, and vascular occlusion. Various drugs had been used for the treatment of ISSNHL, including vasodilators, diuretics, anticoagulants, plasma expanders, corticosteroids, carbogen inhalation, vitamins, and combined therapies. The evidence supporting effectiveness of these treatments is poor. Many reasons for the lack of clinically effective treatments have been postulated, including a relatively high rate of spontaneous recovery and a paucity of well-designed randomized controlled trials. The use of steroids in the treatment of ISSNHL is probably the most widely studied. While there is good evidence to suggest that the use of intratympanic steroids is effective, at least in the salvage situation, no compelling evidence is found to support efficacy of systemic steroids (reviewed in Stachler et al. 2012). This seemingly incongruous finding may reflect insufficient delivery of drug to the cochlea with oral administration.

Reactive oxygen species (ROS) have been shown in animal studies to play an important role in cochlear hair cell damage from etiologies such as cochlear ischemia, noise trauma, presbycusis, meningitis-associated hearing loss, radiation, aminoglycoside, and cisplatin ototoxicity (Seidman and Vivek 2004; see also many chapters of this book). The main sources of ROS production within the cochlea appear to be hair cell and supporting cell mitochondria, or enzymes such as xanthine oxidase and nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase). Once generated, ROS are responsible for direct cellular damage to lipids, proteins, and DNA (Ciorba et al. 2010). ROS also cause oxidative stress which promotes apoptosis by affecting mitochondrial permeability, release of cytochrome *c*, and activation of p53 and caspases (Cheng et al. 2005; Devarajan et al. 2002). It is conceivable that, as in the other known etiologies, oxidative stress resulting in cochlear cellular damage might be important in ISSNHL. If this hypothesis is true, antioxidants should be effective in the treatment of ISSNHL.

21.1.2 Role of Reactive Oxygen Species

Ciorba et al. (2010) studied the role of ROS in humans with sensorineural hearing loss (presumably reflecting cochlear hair cell pathology) from various etiologies. Ninety-eight samples of perilymph were collected from patients suffering profound sensorineural hearing loss, during cochlear implantation surgery. Seven control

samples were obtained from patients with otosclerosis with spontaneous leakage during stapedotomy surgery. The presence of ROS was demonstrated using spectrophotometric analysis and polyacrylamide gel electrophoresis. The study found the average level of superoxide, a biologically relevant ROS, in the study group to be 27.34 μmol/mg of total protein, vs. 0.36 in the control group. The authors attributed the difference to free radical formation associated with previous hair cell damage in the Organ of Corti.

Capaccio et al. (2012) further investigated the role of oxidative stress specifically in ISSNHL patients, by assessing the balance between ROS production and total antioxidant defense capacity. [how 'bout adding one sentence about how "total antioxidant defense capacity" is measured…likely not common knowledge….or is that my naivety? I agree with this suggestion.] Serum ROS concentrations and total antioxidant capacity were measured by spectrophotometric methods using commercial kits on F.R.E.E. analyzer (Diacron International, Italy). A total of 39 patients with ISSNHL and 70 healthy controls were studied. Not only did the ISSNHL patients have higher serum levels of ROS, they also demonstrated a greater percentage of ROS within the pathological range (64 % vs. 48 %). Total antioxidative capacity was not statistically different between the two groups. These investigators speculated that the hearing impairment in ISSNHL resulted from the inability of the endogenous antioxidant defense system to handle an abrupt rise in ROS.

21.1.3 Antioxidant Treatment in ISSNHL

A number of studies have investigated the possible benefit of antioxidant treatment in ISSNHL. Studies demonstrating significant posttreatment hearing improvements of at least 25 dB or 50 % compared with pretreatment levels are reviewed below.

In a prospective double-blind study, Joachims et al. (2003) studied the possible benefits of vitamin E in the treatment of ISSNHL. Vitamin E, present in cellular lipids, is a donor antioxidant that reduces peroxyl radicals and inhibits the propagation of lipid peroxidation. The control group in this experiment was treated with bed rest, prednisolone, intravenous magnesium, and carbogen inhalation. The study group of 33 patients received additional Vitamin E 400 mg twice daily. In the study group, a recovery rate better than 75 % is observed in 78.8 % of subjects. In comparison, the same degree of recovery was found in only 45.5 % of the subjects in the control group.

The therapeutic efficacy of high-dose vitamin C in ISSNHL was also evaluated in a recent prospective single-blind randomized controlled study (Kang et al. 2013). Vitamin C is known to detoxify by scavenging and reducing free oxygen radicals. In a control group of 36 subjects, systemic steroid was administered for 15 days. In the study group ($n=36$), the subjects received additional high-dose intravenous vitamin C (200 mg/kg/day) for 10 days and oral vitamin C (2,000 mg) for 30 days after discharge. One month after treatment, the study group showed significantly greater complete and partial recovery. Moreover, the complete recovery rate in the study group was more than twice that of the control group.

Another prospective randomized study investigated zinc supplement in the treatment of ISSNHL (Yang et al. 2011). Thirty-three subjects (control group) were given corticosteroid treatment and 33 (experimental group) were given corticosteroids plus oral zinc gluconate (2 tabs twice a day for 2 months). Recovery of hearing was found to be significantly better in the experimental group. A significant correlation between serum zinc level changes and percentage of recovery was also observed. Besides its antioxidant function, zinc has multiple additional functions including a role in modulating auditory neural transmission and anti-inflammatory effects. The authors speculated that zinc was important as an antioxidant for the normal function of the cochlea as it is an essential component of Cu/Zn superoxide dismutase in the stria vascularis.

In China, Xiong et al. (2012) studied Radix astragali (RA) in the treatment of ISSNHL. According to the authors, RA is a traditional Chinese herb widely used in treating ROS-mediated injury in various organs. Its antioxidant properties have been postulated in Chinese medical history and RA has reportedly been used in the treatment of deafness for hundreds of years. In a case-control study, the authors compared the hearing gains in 46 ears treated with RA (given intravenously daily for 10 days) with 46 control ears not treated with RA. The recovery of hearing in the RA-treated group was significantly better than in the control group.

While most antioxidant studies in ISSNHL patients have been based only on pure-tone hearing averages over the speech range, Angeli et al. (2012) extended his study to the upper end of the speech frequency range (at 4,000 Hz). In a case-control study, adult patients with ISSNHL were treated with oral prednisone plus intratympanic dexamethasone either alone or in combination with oral antioxidant L-*N*-acetylcysteine (LNAC) at a dose of 1,200 mg three times daily for 2 weeks. LNAC is an antioxidant which besides its nucleophilicity and redox interaction, is also readily deacetylated in cells to yield L-cysteine and thereby promote intracellular glutathione (GSH) synthesis (Low et al. 2008). The investigators reported that combination therapy corticosteroids plus LNAC was associated with improved hearing over corticosteroids alone, particularly at the 6-month follow-up and at 4,000 Hz.

The above studies suggest that antioxidants have an effect on the recovery of ISSNHL. It can be argued however, that antioxidants have multiple functions and that the benefits observed may not necessarily be a result of their antioxidant effects. Nevertheless, taken together, and especially in the light of laboratory studies demonstrating the importance of ROS in cochlear hair cell damage, these data do indicate that antioxidant treatment of patients with ISSNHL will support their recovery of hearing. This suggestion is further substantiated by the observation that the antioxidant LNAC enhanced recovery of higher frequency hearing in ISSNHL (Angeli et al. 2012).

21.1.4 ISSNHL Affecting Higher Frequency Thresholds

It is well known that permanent sensorineural hearing loss from most etiologies (e.g., presbycusis, noise-induced, drug-induced, and radiation) tends to affect the high frequencies more than lower frequencies. Reflecting the tonotopic distribution

of hair cells in the cochlea, the tendency for higher frequency sounds to be affected implies that hair cells in the basal coil are more susceptible than cells in the apical region. Indeed, this pattern of differential cochlear damage had been observed histologically in cisplatin (Hinojosa et al. 1995). Various theories have been advanced to explain the preferential destruction of the basal outer hair cells (OHC) (Wu et al. 2002). One theory invokes a pharmacokinetic explanation, in that drugs gain access to the cochlea from the basal turn. However, the gradient of loss is observed in cochlear explants where applied drugs have equal access to all portions of the cochlea (Richardson and Russell 1991). Another explanation offered suggests a role of efferent innervation of OHCs, which is higher in the base than in the apex. This argument is also not tenable, given the preservation of the differential sensitivity in explant cultures, which lack efferent innervation to the hair cells.

A third potential explanation of the observed base-to-apical gradient in OHC sensitivity to ototoxic substances may be related to ROS generation. In a study on aminoglycoside ototoxicity, OHC deaths in the Organ of Corti were observed to follow a base-to-apex gradient (Sha et al. 2001). The gradient of survival was eliminated and basal hair cells were preserved by the addition of various antioxidants, implying that the accelerated death of basal hair cells was due to damage by ROS. This argument is supported by the observation that apical OHCs have much higher levels of GSH than basal OHCs (Rybak and Whitworth 2005). The base-apex gradient observed in ototoxicity may, therefore, be due to an intrinsic susceptibility to free radicals that differs among cochlear cell populations.

Human studies also support a regional-specific susceptibility to oxidative stress in the cochlea. Radiation (Low et al. 2009) and cisplatin (Rybak et al. 2007) use has been postulated to produce synergistic ototoxicity. Low et al. (2006) demonstrated that patients who received radiotherapy with concurrent/adjuvant chemotherapy for treatment of nasopharyngeal carcinoma experienced greater sensorineural hearing loss than those treated with radiation therapy alone, particularly in the high frequency range. They postulate that this hair cell pathology is secondary to synergistic ROS-related mechanisms produced by radiation-induced and chemotherapy-induced free radical formation. The efficacy of the antioxidant LNAC in the prevention of noise-induced hearing loss has been studied; early results have been promising, demonstrating greater effect at high frequencies (Lin et al. 2010). Moreover, LNAC prevented gentamicin-induced hearing loss in hemodialysis patients, with greater protection afforded at higher frequencies (6,000–12,000 Hz; Feldman et al. 2007).

The prognosis of ISSNHL is known to be more favorable in patients with upsloping audiograms, compared to those with more high-frequency hearing loss. This may well have something to do with different pathophysiological processes involved in ISSNHL, with ROS-induced irreversible damage of basal cochlear hair cells playing a greater role in patients with high-frequency hearing loss. In clinical practice, recovery in low and mid frequency hearing is often better than that in the higher frequencies after steroid therapy (see Figs. 21.1 and 21.2). Hence, the observation that LNAC treatment in ISSNHL conferred preferential frequency-based benefits favoring the higher frequencies is consistent with the hypothesis that an altered oxidative status contributes to the cochlear injury regardless of the presumed etiology.

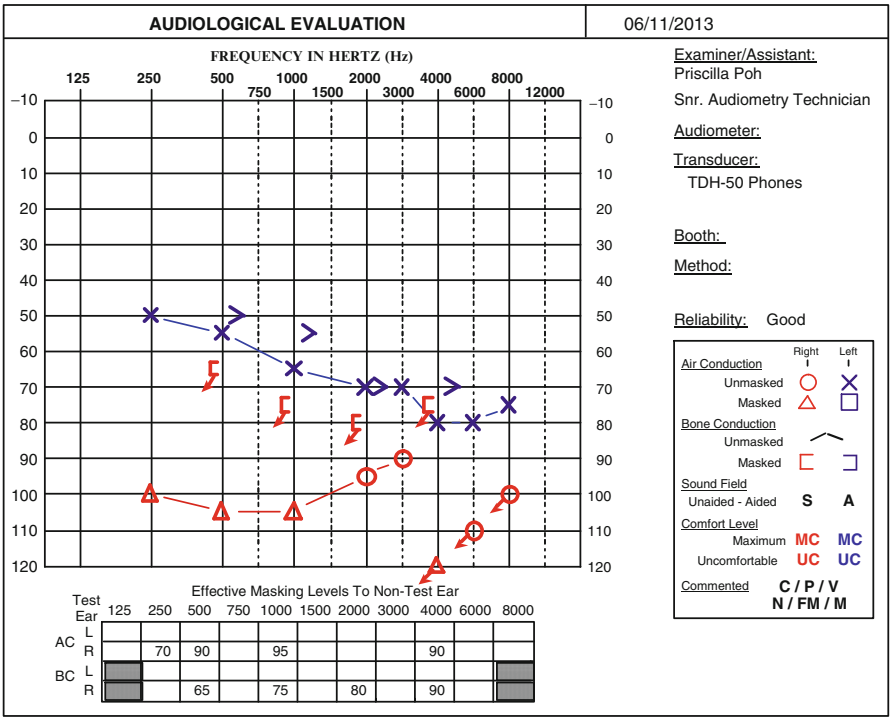

Fig. 21.1 Pretreatment pure-tone audiogram of a patient presenting with right profound sudden sensorineural hearing loss

21.1.5 Combined Steroid and Antioxidant Treatment in ISSNHL

Based on the potential therapeutic effects of steroids and antioxidants, the addition of antioxidants to steroid treatment may yield better outcomes in ISSNHL. Oxidative stress is known to be a key determinant in dysfunction of the vascular endothelium (Capaccio et al. 2012). Beneficial effects of steroids are thought to be due to their anti-inflammatory properties. Steroid-responsive hearing disorders may also be attributed to vascular disruption in the stria vascularis, which may lead to a breakdown of the blood-labyrinth barrier and endolymph ion homeostasis (Trune and Canlon 2012). Hence, antioxidants and steroids may well have complementary or even synergistic effects in improving vascular function. On the other hand, although glucocorticoids confer antiapoptotic properties in the hair cells, there is only weak

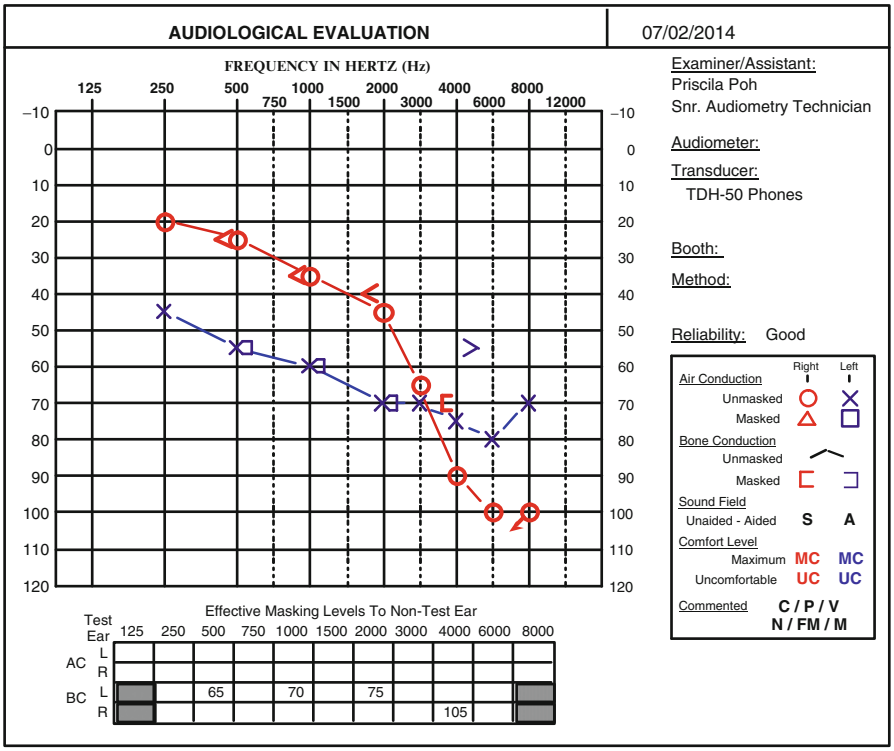

Fig. 21.2 Posttreatment audiogram of the same patient treated with a course of intratympanic methylprednisolone showing good recovery in the low and mid (but not the higher) speech frequencies

expression of glucocorticoid receptors in OHCs (Trune and Canlon 2012; Amsterdam et al. 2002). Therefore, the prevention of apoptosis of OHCs might be better effected by antioxidants than by steroids.

21.1.6 Conclusion

The pathogenesis of ISSNHL and its treatment are controversial, although there is now good evidence to support the use of steroids delivered by the intratympanic route. In recent years, there has been a growing body of evidence to suggest that free oxygen radicals play an important role in ISSNHL and that antioxidant treatment might be of benefit. Further well-designed randomized blinded studies are warranted to find out if the early addition of antioxidants to standard steroid therapy can enhance recovery of ISSNHL, particularly in the less treatment-responsive high-frequency hearing losses.

21.2 Meniere's Disease

Meniere's disease (MD) is an inner ear disorder characterized by spontaneous episodes of vertigo lasting at least 20 min, accompanied by fluctuating low-frequency sensorineural hearing loss, aural fullness, and tinnitus. Endolymphatic hydrops (ELH) was first noted in the temporal bones of patients suffering from Meniere's syndrome in 1938. However, recent work has shown that ELH is a histologic marker of MD rather than the direct pathophysiologic cause (Merchant et al. 2005). Dysregulation of endolymph, infectious, autoimmune, vascular, genetic, dietary, endocrine, and many other etiologic factors have been hypothesized to result in ELH (Kiang 1989). The membrane potential of sensory cells found within the inner ear is regulated by K^+ selective channels and the cycling of K^+ from endolymph to perilymph and back helps to generate the electrochemical gradient needed for sensory transduction (Wangemann 2002). Fibrocytes within the cochlea are involved in this K^+ cycling. In surgically blocked endolymphatic sacs of guinea pig, type 1 fibrocytes showed osmotic stress with an increase in Na–K–2Cl cotransporter (NKCCl) and decrease in taurine and C-Jun *N*-terminal kinase (JNK) (Semaan et al. 2005; Shinomori et al. 2001).

While a major pathophysiologic theory of MD etiology invokes rupture of the membranous labyrinth with resultant mixing of endolymph and perilymph and end organ damage, ultrastructural evidence instead points to neuronal injury, lending credence to the theory of neurotoxicity (Semaan et al. 2005). There is increasing evidence that oxidative stress plays a critical role in pathogenesis of MD.

Nitric oxide (NO) is generated by nitric oxide synthase (NOS). Type 1 (brain/neuronal) and Type 3 (endothelial) NOS are activated by calcium-ion influx, are constitutive, and produce small amounts of NO. In contrast, Type 2 (inducible) NOS is calcium ion independent, and can produce 100–1,000-fold more NO than the other types. Nitric oxide has been noted in afferent nerve endings, inner and OHCs, the organ of Corti, stria vascularis, spiral ligament, and the spiral vessel to the basal membrane in the guinea pig model (Shi et al. 2001). Injection of keyhole limpet hemocyanin into the endolymphatic sac of guinea pigs simulated ELH and revealed that inducible Type 2 NOS is found within the hydropic vestibule, especially in the sensory epithelium and vestibular ganglion cells (Watanabe et al. 2001). NO release results in increased release of excitatory amino acids such as glutamate; these peaks of NO release are present during both early and late phase of tissue damage (Takumida and Anniko 2002). Guinea pig models have implicated glutamate in ELH, with upregulation of NOS and caspase (Anne et al. 2007). In general, NO generation results in glutamatergic activation of *N*-methyl-D-aspartate (NMDA) receptors. Chronic glutamate overstimulation of the NMDA receptor leads to calcium influx, with the ensuing cellular stress leading to activation of the caspase cascade and NO production (Orrenius et al. 2003). Loss of type 1 spiral ganglion neurons through glutamate excitotoxicity has been implicated as the main pathologic finding with hearing loss found in ELH (Megerian 2005).

Stress-induced ROS, free radicals, and caspase can be measured in the spiral ganglion cells, stria vascularis, fibrocytes, organ of Corti, and blood vessels of the spiral ligament in surgically hydropic guinea pigs (Labbe et al. 2005). The resultant damage to mitochondrial membrane potential leads to cytochrome *c* release and cleavage of procaspase-9 and further downstream activation of caspase-3 (Van De Water et al. 2004, see also Chap. 19, by Van De Water). While caspase-9, caspase-3, and to some extent caspase-8, are activated through this intrinsic mitochondrial cell death pathway and participate in the normal development and maturation of the membranous labyrinth and cochleovestibular ganglion, post development, they are also implicated in the cause of cell death and propagation of ELH. High volumes of ROS lead to programmed cell death (Bras et al. 2005).

Cytoprotection from ROS are represented by vitagenes, including heat shock proteins (Hsps) heme-oxygenase 1, and Hsp-70 (Calabrese et al. 2010). There is ongoing research to explore intrinsic capabilities of GSH to control redox homeostasis along with exogenous medications to control oxidative stress within the inner ear (Abi-Hachem et al. 2010). Riluzole, a glutamate release inhibitor, originally developed to treat amyotrophic lateral sclerosis (Hugon 1996), has been shown to at least reduce hearing loss related to acoustic trauma (Wang et al. 2002). Dimethylsulfoxide (DMSO), a potential carrier for riluzole, independently and reversibly inhibits NMDA, suppresses calcium influx produced by glutamate and scavenges ROS, which can prevent apoptosis (Lu and Mattson 2001). Melki et al. (2010) used the *Phex*$^{Hyp\text{-}Duk}$ mouse, which spontaneously develops ELH and postnatal hearing loss to study possible protective effects of riluzole and DMSO. Mice were treated, beginning at postnatal day 6, with either riluzole and DMSO (required carrier), DMSO alone, or no-injection control. The authors demonstrated a statistically significant protective effect against hearing loss with DMSO, independent of riluzole, at least in the dosage used. DMSO has antioxidant properties and its potential sites of action are illustrated in Fig. 21.3 (Melki et al. 2010).

21.2.1 Conclusion

Evidence shows that while glutamate is an important excitatory amino acid within the inner ear, glutamate excitotoxicity results in increased oxidative stress. This initiates downstream apoptotic processes within type 1 spiral ganglion cells which may be responsible for eventual irreversible hearing loss in Meniere's disease. While more research is needed, there is hope that antioxidants may prevent or reduce permanent hearing loss associated with this disorder. This has great potential clinically as therapeutic hearing preservation has so far been an elusive goal in the management of Meniere's disease. Given the current level of evidence of possible efficacy and the safety profile of many of the antioxidants available, clinical trials to assess their utility would seem to be warranted at this time.

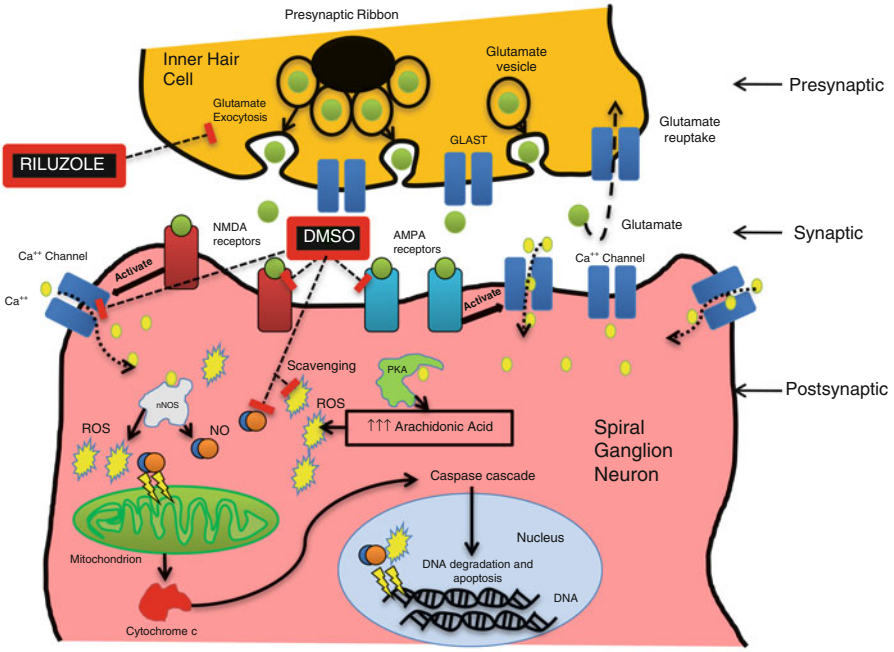

Fig. 21.3 Glutamate excitotoxicity pathway and potential site of action of riluzole and DMSO. Riluzole acts by blocking glutamate release and DMSO by antagonizing glutamate's action by inhibiting NMDA and AMPA receptors and diminishing the calcium influx into the cell by (hypothetical) modification of the characteristics of the calcium channels. DMSO also acts as a scavenger of ROS. *PKA* protein kinase A; *nNOS* neuronal nitric oxide synthase; *NO* nitric oxide (reproduced from Melki et al. 2010 with permission)

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Part IX
Head and Neck

Chapter 22
Role of Free Radicals in Head and Neck Cancer

Carter Van Waes

22.1 Overview

Oxidative stress and free radical production from metabolism by host cells and resident microbiota is a continuous process for which cells have protective antioxidant defenses, which usually mitigate damage to prevent or delay development of cancer (Gorrini et al. 2013). These defenses include sensors of oxidative stress, such as KEAP1, and transcription factor NRF2, that induce detoxification enzymes. In the head and neck, these defenses may be overwhelmed by long-term exposure to tobacco or ultraviolet light carcinogens that cause head and neck squamous cell carcinomas (HNSCC), each arising respectively from the squamous epithelia of the aerodigestive tract or skin. These carcinogens induce free radical-mediated or direct DNA damage that result in cancer initiating mutations (Choudhari et al. 2014). Alterations affecting key tumor suppressor genes such as *TP53*, related oncogene *ΔNp63*, and *PIK3CA*, the PI3kinase catalytic subunit alpha, are prevalent (Walter et al. 2013). These DNA damaging signals and genomic alterations in turn may activate transcription factor Nuclear Factor-κB to promote cell survival and host inflammatory responses (Yang et al. 2011; Vander Broek et al. 2014; Du et al. 2014; Cooks et al. 2013), which further enhance free radical production and cumulative DNA damage, resulting in cancer progression. Mutations in genes such as KEAP1 and NRF2 critical to sensing and inducing antioxidant and survival responses, or the Fanconi/BRCA pathway important in DNA repair, enhance susceptibility to HNSCC (Walter et al. 2013, Van Waes 2005). Anti-inflammatory drug celecoxib in

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combination with Epidermal Growth Factor Receptor inhibitor erlotinib has demonstrated activity in premalignant lesions. In genetically engineered experimental animal models with genetic defects in Fanconi D2/TP53 or TGFβ receptor 1/Pten genes that activate PI3K signaling, synthetic antioxidants, or PI3K inhibitors may delay onset of cancer, and have clinical translational potential (Zhang et al. 2008; Herzog et al. 2013).

22.2 Tobacco-Related Free Radical Damage

Development of HNSCC is most frequently associated with exposure to tobacco products, and further enhanced when combined with alcohol. Tobacco smoke and smokeless tobacco contain nitrosamine and polyaromatic hydrocarbon carcinogens whose electrophilic metabolites induce reactive oxygen species (ROS) and reactive nitrogen species (RNS) that modify or disrupt DNA, as well as form direct DNA adducts whose faulty repair cause mutations (Fig. 22.1) (Choudhari et al. 2014; Hecht 2012). One of the major consequences of tobacco and smoke metabolites or induced ROS is increased formation of 8-hydroxy-deoxyguanosine (8-OHdG), which is potentially mutagenic (Hecht 2012). In parallel, chronic nicotine and carcinogen exposure can induce PI3K-Akt and PKA signal activation of transcription factor NF-κB (Fig. 22.1), which promotes cell survival and proliferation, and additional ROS production by infiltrating inflammatory cells, exposing progeny to cumulative mutations (Hecht 2012; West et al. 2004; Tsurutani et al. 2005; Dennis et al. 2005). As a result, tobacco metabolite- and ROS-related mutations across the genome are frequent, and cumulatively affect key tumor suppressor genes and oncogenes, resulting in autonomous loss of growth control, genomic instability, ROS homeostasis, and malignant transformation.

22.3 Activation of NF-κB and Inflammation-Related Free Radical Damage

The NF-κB/REL family transcription factors are aberrantly activated in HNSCC and other cancers, and critically promote cell survival, inflammation, and angiogenesis (Fig. 22.1) (Van Waes 2007). As aforementioned, nicotine and tobacco metabolites can promote PI3K-Akt and PKA signaling, and my laboratory showed that PI3K and PKA contribute to aberrant transactivation of NF-κB observed in HNSCC (Fig. 22.1) (Bancroft et al. 2002; Arun et al. 2009). Additionally, many injury and pathogen inducible signal pathways converge to activate NF-κB (Van Waes 2007). Carcinogen and ROS-induced DNA damage can promote sumoylation and activation of Inhibitor-κB kinases (IKKs), which mediate NF-κB nuclear translocation and activation. Further, ROS can promote degradation of ubiquitin ligase KEAP1,

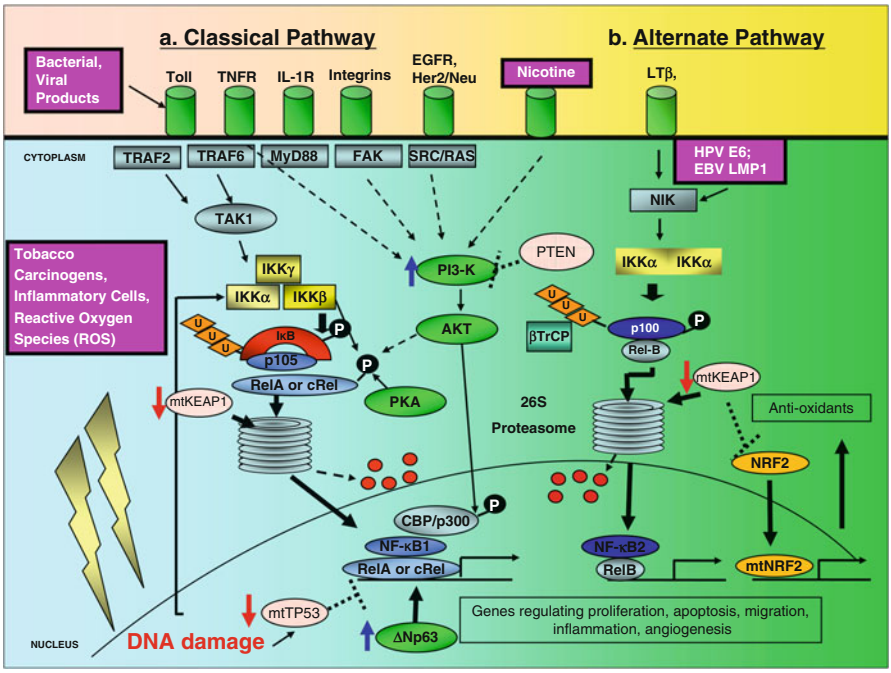

Fig. 22.1 Tobacco carcinogen and inflammatory response-induced reactive oxygen species (ROS), bacterial, and viral products mediate inducible and genomic alterations in the PI3K-NF-κB, TP53/63, and KEAP1/NRF2 pathways in HNSCC. (**a**) Tobacco carcinogen and inflammatory cell-induced ROS cause DNA damage and degrade sensor and ubiquitin ligase KEAP1, inducing activation of the classical Inhibitor-κB kinase (IKK)-NF-κB pathway, which elicits transcription of cancer promoting genes. Carcinogen and ROS-induced genomic mutations in tumor suppressor TP53 and amplifications causing overexpression of oncogenic family member ΔNp63 and PI3-Kinase (PI3K) result in loss of growth control and enhance NF-κB signaling. Classical NF-κB activation may be enhanced by bacterial and viral products, inflammatory and growth factors. (**b**) Alternative NF-κB pathway activation (and TP53 inactivation) can be directly mediated by HPV E6 and EBV LMP1 oncogenes. ROS-induced KEAP1 and NRF2-mediated transcription of endogenous antioxidants may also be compromised by genomic alterations

enhancing IKK-mediated signaling (Fig. 22.1). Bacterial, human papilloma virus (HPV) and Epstein Barr Virus (EBV) pathogens have also been implicated in development of HNSCC, and can induce activation of Toll-Like Receptor, IKKs, and alternative pathways that promote NF-κB activation (Van Waes 2007; James et al. 2006; Szczepanski et al. 2004) (Fig. 22.1).

The consequences of such chronic injury-induced signal activation of NF-κB are pathologic. NF-κB promotes expression of *Cyclin D1* and *BCL-XL* genes that promote cell proliferation and survival of HNSCC cells (Van Waes 2007; Lee et al. 2008; Duan et al. 2007). NF-κB also promotes expression of angiogenesis factors *IL-6*, *IL-8*, *GRO1*, and *VEGF* (Duffey et al. 1999; Bancroft et al. 2001; Loukinova et al. 2001) that recruit and activate monocytic and myeloid inflammatory cells

(Loukinova et al. 2000; Young et al. 2001). Activated myeloid-derived cells produce ROS, which likely further exacerbates cell and DNA damage, related signaling and mutations, and compromises immune defenses to malignant cells (Kotsakis et al. 2012; Vasquez-Dunddel et al. 2013).

22.4 Role TP53 and p63/PI3KCA Genetic Alterations in Genomic Instability and Inflammation in HNSCC

Among genetic alterations, mutation or deletion of TP53 is the most frequent, occurring in over 70 % of 279 HNSCC tumors studied as part of The Cancer Genome Atlas (TCGA) (Fig. 22.2) (TCGA Network 2015). TP53 is a ROS and DNA damage inducible transcription factor that mediates growth arrest and DNA damage repair, or death of cells with irreversibly damaged DNA (Fig. 22.1). Hence, TP53 serves as the "Guardian of the genome," and its loss leads to uncontrolled proliferation, genomic instability, and progressive genomic alterations (Lane 1992; Stiewe 2007). Among the gains, amplification of the locus containing the gene encoding a *TP53* family oncogene *ΔNp63*, and amplification or activating mutations of *PIK3CA*, the PI3kinase catalytic subunit alpha, are prevalent (Fig. 22.2) (Walter et al. 2013). In ~20 % cases, *ΔNp63* and *PIK3CA* are included in the same amplicon, while overall, *PIK3CA* is amplified or mutated in 36 % of cases. Interestingly, these genomic alterations in *TP53*, *ΔNp63*, and *PIK3CA* may contribute to inactivation of TP53-dependent responses, and constitutive activation of transcription factor Nuclear Factor-κB, cell survival, and host inflammatory responses (Fig. 22.1) (Yang et al. 2011; Vander Broek et al. 2014; Du et al. 2014; Cooks et al. 2013), that further enhance free radical production and cumulative DNA damage, resulting in cancer progression.

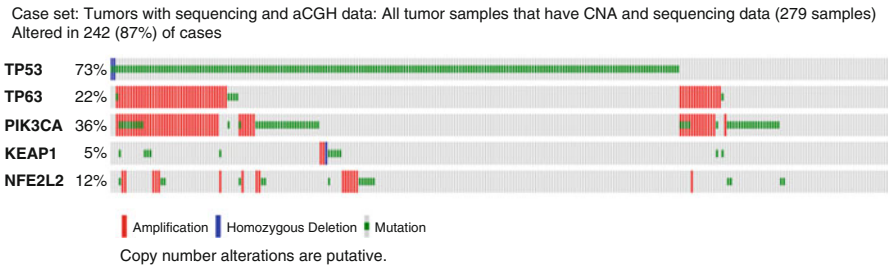

Fig. 22.2 Genomic alterations in TP53, TP63, PIK3CA, KEAP1, and NFE2L2 genes in HNSCC. Publically available data for the genes indicated were queried from 279 head and neck squamous cell carcinomas from The Cancer Genome Atlas (TCGA) using cbioportal (http://www.cbioportal.org/public-portal/). TP53 is mutated in over 70 % of tumors. The adjacent loci containing TP63 and PI3K catalytic subunit PIK3CA are co-amplified in ~20 % of HNSCC, and activating mutations in PIK3CA are observed in additional tumors. Mutations in the oxidative stress pathway including KEAP1 mutations (~5 %) and amplification or mutation of NRF2 (~12 %) are found. Key, *red bars*, amplifications; *blue bars*, homozygous deletion; *green bars*, mutations

22.5 Role of KEAP1/NRF2 Genetic Alterations in HNSCC Susceptibility

KEAP1 is an important sensor of oxidative stress, and ubiquitin ligase, which in the absence of stress binds and promotes proteasomal degradation of IKKβ proteins, inhibiting NF-κB activation and cell survival, and of transcription factor NRF2, inhibiting antioxidant genes (Fig. 22.1) (Tian et al. 2012). In the presence of ROS, KEAP1 cysteine residues undergo conformational changes that promote IKK-induced NF-κB activation and cell survival, while releasing NRF2 for nuclear translocation and activation of antioxidant genes. The antioxidant genes include glutathione-S-transferases (GSTs), NADP(H) quinone oxidoreductase (NQO1), catalase, and superoxide dismutases (SODs), important in neutralizing ROS. In HNSCC, mutations of KEAP1 are observed in ~5 % and in NRF2 are observed in ~12 % of HNSCC (Fig. 22.2), suggesting genomic alterations affect KEAP1 regulated NF-κB prosurvival signaling and NRF2 antioxidant responses in a subset of HNSCC. Most HNSCC tumors with alterations in KEAP1 and NRF2 also appear to have undergone mutations in TP53 (Fig. 22.2). Studies in transgenic mouse models suggest NRF2 may inhibit initiation of tumorigenesis, but enhance progression of established tumors (Satoh et al. 2013). This observation involving NRF2 and endogenous antioxidants mirrors the cautionary observation that antioxidant β-carotene can inhibit initiation in preclinical models of lung cancer, while enhancing progression and mortality in smokers (ATBC 2003), who could have had premalignant lesions with TP53 mutations.

22.6 Role of Alterations in Fanconi/BRCA DNA Damage Response in HNSCC Susceptibility

The Fanconi Anemia (FANC) and Breast/ovarian cancer (BRCA) genes and proteins are now known to comprise a pathway critical in mediating repair of ROS-mediated DNA damage by nonhomologous recombination (Kee and D'Andrea 2012). Overall, the pathway includes 15 FANC genes, BRCA1 and BRCA2. Mutations in FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL, and FANCM account for approximately 90 % of patients. These result in loss of FANCD2 and FANCI monoubiquitylation, the key regulatory event in the FA pathway. Besides loss of DNA repair, FANCD2 activates transcription of a TP53 homologue TAp63 that suppresses tumorigenesis (Park et al. 2013). Patients with FANC mutations are prone to bone marrow failure with anemia and leukemia in childhood, or development of HNSCC and genitourinary tract SCCs in young adulthood. While BRCA gene mutations predispose to breast and ovarian cancer, they have also been detected in patients with HNSCC. Overall, genomic alterations in FANC and BRCA genes are detected in 86/279 (~31 %) of HNSCC tumors in TCGA (Fig. 22.3). The HNSCC that arise in patients with FA patients in their 20–40 s frequently occur

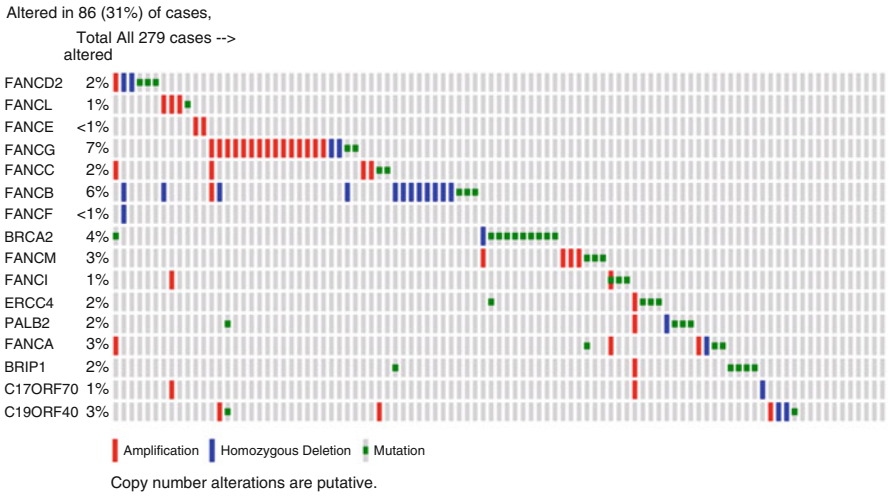

Fig. 22.3 Genomic alterations in Fanconi and BRCA genes in HNSCC. Publically available data for the genes indicated were queried from 279 head and neck squamous cell carcinomas from The Cancer Genome Atlas (TCGA) using cbioportal (http://www.cbioportal.org/public-portal/). Fanconi genes include named FANC, and other genes listed. Most genes exhibit deletions or inactivating mutations, but FANCG is more often amplified, which could enhance or repair of ROS-mediated genomic instability, respectively. Key, *red bars*, amplifications; *blue bars*, homozygous deletion; *green bars*, mutations

in the absence of tobacco use, and occur in the oral cavity in the tongue and mucosa adjacent to areas of exposure to dental trauma and microbiota (Van Waes 2005). The FA pathway has recently been shown to limit human papilloma virus replication and transformation by the HPV E7 gene (Hoskins et al. 2012; Park et al. 2010). However, the extent of the role of HPV in FA HNSCC remains unclear, as others report that the mutational spectrum in HNSCC in FA includes genes such as TP53, similar to that in tobacco-related HNSCC (van Zeeburg et al. 2008). Increased oxidative stress and potential for mutations and malignant transformation has been detected in FA cells (Du et al. 2008), and is enhanced by inflammatory signaling and induction of ROS by TNFα (Li et al. 2007).

22.7 Potential of Anti-inflammatory Agents, Antioxidants, and PI3K-mTOR Inhibitors to Delay Malignant Progression and for Clinical Translation

Based on the potential role of HNSCC-associated inflammation and ROS in promoting HNSCC, anti-inflammatory drugs have been of interest. Many anti-inflammatory drugs inhibit NF-κB or NF-κB targets such as Cyclooxygenases, responsible for inflammatory prostaglandins (Van Waes 2007). Proteasome inhibitors preventing

IκB degradation and NF-κB activation and inflammation yielded incomplete and transient responses in preclinical and clinical trials, which were found to be due to compensatory activation of other prosurvival signaling pathways (Allen et al. 2008; Chen et al. 2008). Cyclooxygenase inhibitor ketorolac inhibited inflammatory cells in response to HNSCC in preclinical studies, but showed a similar 30 % response rate as placebo in reducing leukoplakia (Hong et al. 2000; Mulshine et al. 2004). However, Cyclooxygenase 2 plus Epidermal Growth Factor Receptor inhibitors were found to synergistically inhibit head and neck squamous cell carcinoma tumorigenesis in preclinical and clinical studies (Saba et al. 2014). In a phase I study with a combination of COX2 inhibitor celecoxib and EGFR inhibitor erlotinib in patients with advanced premalignant lesions, the overall histologic response rate was 63 % (complete response 43 %, partial response 14 %, stable disease 29 %, disease progression 14 %). With median follow-up of 36 months, mean time to progression to higher-grade dysplasia or carcinoma was 25.4 months. Encouraging responses to the celecoxib and erlotinib combination correlated with EGFR pathway inhibition, where downregulation of EGFR and p-ERK in follow-up biopsies correlated with response to treatment (Vander Broek et al. 2013).

With evidence for a relatively high prevalence of PI3K-mTOR pathway alterations HNSCC, and their importance in activation of NF-κB and inflammatory responses (Vander Broek et al. 2013), PI3K and mTOR inhibitors have been the subject of preclinical and clinical investigation. In genetically engineered experimental animal models with genetic defects in TGFβ receptor 1/Pten genes and activated PI3K signaling, a synthetic PI3K-mTOR inhibitor delayed onset of HNSCC, demonstrating clinical translational potential (Herzog et al. 2013). In a clinical trial of mTOR inhibitor rapamycin underway at NIH, clinical responses have been observed in patients with stage II–IV oral and oropharyngeal cancers [C. Van Waes, unpublished observations].

Based on the hypothesis that FA cells are more prone to oxidative damage, we examined and demonstrated an increase in ROS DNA marker 8-OHdG in human FA fibroblast lines relative to control cell lines (Zhang et al. 2008). A synthetic nitrosamine antioxidant tempol reduced 8-OHdG similar to normal levels in these FA cells, and cells from Fancd2 knockout mice. Fancd2−/− Trp53+/− mice on a tempol diet showed a significantly longer mean tumor-free survival (mean = 390 days) than the mice on placebo diet (mean = 308 days) ($P < 0.01$). After early deaths due to leukemias, statistical analysis revealed that tempol treatment significantly increased the mean epithelial tumor-free survival time by 38 % in Fancd2−/− Trp53+/− mice ($P < 0.0001$). These data suggest that tempol may have a role in reducing oxidative DNA damage and malignant transformation in FA (Zhang et al. 2008), although naturally occurring antioxidant resveratrol or n-acetylcysteine did not have significant chemopreventive effects in the same model (Zhang et al. 2014).

In conclusion, anti-inflammatory, antioxidants, and PI3K-mTOR inhibitors targeting specific genetic alterations have preclinical or clinical activity and potential for further clinical investigation in prevention of HNSCC.

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Chapter 23
Free Radicals and Sleep Apnea

Muzaffer Kanlikama and Elif Baysal

Abbreviations

Ang-II	Angiotensin II
AP-1	Activator protein-1
Aryl	Arylesterase
Cp	Ceruloplasmin
CPAP	Continuous positive airway pressure
CRP	C-reactive protein
ET-1	Endothelin-1
HIF-1	Hypoxia-inducible factor-1
hsCRP	High-sensitivity C-reactive protein
IL	Interleukin
LOOH	Lipid hydroperoxide
MMP-9	Matrix metalloproteinase-9
NF-κB	Nuclear factor kappa B
NOx	Nitric oxide derivatives
ODI	Oxygen desaturation index
OSAS	Obstructive sleep apnea syndrome
PON	Paraoxonase
ROS	Reactive oxygen species
Sp-1	Sp-1 transcription factor
TAS	Total antioxidant status

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TEAC Trolox equivalent antioxidant capacity
TNF Tumor necrosis factor
TOS Total oxidant status

23.1 Introduction

Oxidative stress is defined as the imbalance between the production and elimination of reactive oxygen species (ROS) or free radicals, which are molecules that have unpaired electrons that tend to react chemically (Lavie 2009). These molecules are produced during normal cellular activities and are eliminated by enzymatic or nonenzymatic reactions called antioxidant systems. An imbalance between the production and elimination of ROS can lead to pathological conditions such as inflammatory diseases and many metabolic diseases (Lavie 2009).

Free radicals are found under normal physiological conditions and are beneficial when produced at low levels. However, they are harmful when the endogenous antioxidant defense systems are overwhelmed (oxidative stress) (Rahman 2007; Lavie 2003). Several studies, however, support a role for oxidative stress in obstructive sleep apnea syndrome (OSAS). Increased production of ROS, activation of redox (Lavie 2009)-sensitive gene expression, is suggested by the literature (Lavie 2003). Redox sensitive genes productions include VEGF, erythropoietin, endothelin-1, inflammatory cytokines and adhesion molecules that reveal the participation of redox-sensitive transcription factors as hypoxia-inducible factor-1 (HIF-1), activator rotein-1 (AP-1), and nuclear factor kappa B (NF-κB), increased adhesion mole cule-dependent increased activity combined with diminished NO bioavailability, result with endothelial cell damage and dysfunction (Lavie 2003).

Antioxidant enzymes and vitamins protect the organism from their potential for cellular damage (Barcelo et al. 2006).

The literature describes several ROS generating systems: the NADPH oxidase, xanthine oxidase, mitochondrial chain, and uncoupled nitric oxide synthase systems. These contribute to endothelial dysfunction, atherosclerosis, arterial hypertension, pulmonary hypertension, and heart failure (Dumitrascu et al. 2013).

OSAS is characterized by recurrent obstruction of the upper airway, which usually results from oxygen desaturation and leads to arousal from sleep. OSAS is the second most common respiratory system disorder, and the prevalence of OSAS in adults is 2–4 % (Christou et al. 2003). The American Heart Association and American College of Cardiology suggest that 85 % of those with OSAS have not been diagnosed (Christou et al. 2003). Patients with OSAS have characteristic symptoms such as excessive daytime sleepiness, chronic fatigue, or neurocognitive decline. The syndrome is usually associated with male sex, middle age, central obesity, smoking, and sedentary lifestyle (Lavie 2009).

In literature there are some controversial points about oxidative stress and OSAS. Barcelo et al. (2006) reported increased systemic oxidative stress in severe cases of OSAS. In contrast, a number of studies propose that there are no differences in the

lipid peroxidation products of OSAS patients and control groups (Baysal et al. 2012; Svatikova et al. 2005; Wali et al. 1998; Alzoghaibi and Bahammam 2005). The role of free radical scavengers in OSAS remains unclear. Christou et al. (2003) showed that patients with severe OSAS have reduced antioxidant capacity values, and a negative correlation was found between antioxidant capacity and disease severity. Recently, additional information about the antioxidant status of OSAS patients has been provided. Barcelo et al. (2006) and Baysal et al. (2012) reported that patients with OSAS had lower total antioxidant status (TAS) levels.

23.2 Etiopathology of OSAS and Oxidative Stress

OSAS is a disorder of hypoxia and reoxygenation episodes (Baysal et al. 2012). Both apneas and hypopneas generally end with arousal and reoxygenation. This can result in the production of ROS. Hypoxia can result in microsomal radical generation and is associated with a loss of the cellular energy metabolites and reductants that protect the cell against radicals from endogenous sources as they are relevant to the physiology and pathophysiology of OSAS (Christou et al. 2003). Free radicals are primarily produced by mitochondria and inflammatory leukocytes, cardiac tissues, and vascular cells as signaling molecules, due to hypoxia/reoxygenation episodes (Lavie 2003).

In OSAS, oxidative stress and inflammation contribute to endothelial dysfunction and atherosclerosis (Del Ben et al. 2012). The association of OSAS and other systemic diseases, such as endocrine, metabolic, and cardiovascular disorders, shows that OSAS is a systemic disease. Recent investigations have revealed that OSA is a risk factor for cardiovascular diseases, including systemic hypertension, myocardial infarction, and cerebrovascular diseases (Htoo et al. 2006; Cofta et al. 2008).

Baysal et al. (2012) found that the level of oxidative stress was increased in patients with OSAS, while the antioxidant capacity was decreased. In patients with OSAS, the levels of antioxidant enzymes such as paraoxonase and arylesterase were also decreased. In addition, OSAS patients have higher levels of lipid peroxide, which is a marker of oxidative stress formed from unsaturated phospholipids, and lower levels of ceruloplasmin, which is a preventive plasma antioxidant.

23.2.1 Pediatric OSAS and Oxidative Stress

In pediatric patients, OSAS causes both oxidative stress and increased activation of inflammation (Gozal 2009). By producing ROS, oxidative stress helps with the initiation of inflammatory events. A second route is endothelial dysfunction via the activation of NADPH oxidase, increased adhesion molecules, and decreased expression endothelial nitric oxide synthase. According to Gozal, monocytes are the primordial cells affected by hypoxia and reoxygenation. In the cardiovascular

consequences of OSAS, proinflammatory CRP and interleukin-6 (IL-6) are important factors (Gozal 2009; Gozal and Kheirandish-Gozal 2008).

The second most important consequence of OSAS is cognitive morbidity. Even habitual snoring results in cognitive problems. OSAS affects the neurocognitive system via inflammation and oxidative stress. Intermittent hypoxia promotes cell apoptosis in the central nervous system. This process of cellular damage due to oxidative stress and lipid peroxidation has been demonstrated in adult rats (Xu et al. 2004; Ramanathan et al. 2005; Burckhardt et al. 2008). A genetic individual susceptibility has also been demonstrated in children. In children with obstructive sleep apnea (OSA) and abnormal cognitive function, the APOE epsilon-4 allele was more common than in children with OSAS and normal cognitive function (Gozal et al. 2007a).

23.2.2 Oxidative Stress and OSAS in Pregnancy

The risk of OSAS increases with obesity, and obesity in pregnancy is associated with adverse outcomes (Fung et al. 2013). The perinatal consequences of OSAS include hypertensive disorders of pregnancy and gestational diabetes. The increased inflammation caused by proinflammatory cytokines leads to the development of these problems. In a study by Fung et al., it is found that even mild OSAS may be associated with significant fetal growth decrements. Good outcomes and the safety of continuous positive airway pressure (CPAP) have been demonstrated in pregnant patients with OSAS (Guilleminault et al. 2004; Wolkove et al. 2008).

23.3 Animal Studies

Sympathetic activation, inflammation, and oxidative stress have major roles in the consequences of OSAS. However, human studies are limited because OSAS patients often have comorbidities and some authors believe that these comorbidities affect the results of oxidative stress and antioxidant markers. Consequently, animal studies and models have shown the role of chronic intermittent hypoxia (Dumitrascu et al. 2013). When rats were exposed to chronic intermittent hypoxia, the reaction to NO decreased, and superoxide dismutase was found to restore coronary vascular reactivity (Tahawi et al. 2001; Phillips et al. 2006). This endothelial dysfunction also affects musculoskeletal arteries (Dopp et al. 2011; El Solh et al. 2006). Nitric oxide levels were found to decrease after CPAP treatment (Ip et al. 2000).

A number of studies have identified several proinflammatory and oxidative stress markers. Some of these and their changes in OSAS are given in Table 23.1.

There is strong evidence supporting the involvement of oxidative stress in the pathophysiology of hypertension. As ROS are mediators of the major physiological vasoconstrictors, like Ang-II and ET-1, OSAS-related hypertension is a result of both an increased carotid chemoreflex and decreased baroreceptor activity (Dumitrascu et al. 2013). Oxidative stress plays an important role in the development

Table 23.1 Studies that have examined oxidative stress in OSAS

Authors	Group	Oxidative stress markers	Changes in oxidative stress markers	CPAP results	Reference
Christou et al. (2003)	Human adults	Antioxidant capacity, TEAC	In severe OSAS, TEAC reduced	Not measured	[5]
Ip et al. (2000)	Human adults	Nitric oxide	Increased synthesis	Not measured	[23, 24]
Volna et al. (2011)	Human adults	Cu, MMP-9, hsCRP, ODI	High positive correlation with markers and OSAS	Not measured	[29]
Gozal (2009)	Children	hsCRP	Positive correlation	Not measured	[26]
Htoo et al. (2006)	Human adults	NF-κB	Positive correlation with OSAS severity	Decreased after CPAP	[8]
Simiakakis et al. (2012)	Human adults	Derivatives of reactive oxygen metabolites, biological antioxidant capacity	No association between markers and OSAS	Not measured	[27]
Baysal et al. (2012)	Human adults	TAS, TOS, PON, Aryl, Cp, LOOH	Increased oxidative stress markers and decreased circulating antioxidant enzymes	Not measured	[6]

and progression of cardiovascular dysfunction associated with hypertensive disease. In addition, disorders in cell functioning can lead to systemic alterations in a chain reaction. Typically, several factors can impair cell function, triggering the same pathophysiological pathway or different ones (Gjorup et al. 2008; Volna et al. 2011; Gozal et al. 2007b; Simiakakis et al. 2012).

23.4 Inflammatory Responses in OSAS

The redox-sensitive transcription factor inflammatory response affects the whole organism. Oxidative stress in OSAS initiates the overexpression of adhesion molecules in blood and endothelial cells. Animal studies have revealed that adhesion molecules are involved in prothrombotic and pre-inflammatory events (Lavie and Lavie 2006; Panes and Granger 1998). An increment in the levels of adhesion molecules can damage the endothelium and promote cytotoxicity against endothelial cells.

23.4.1 Proinflammatory Cytokines

The activation of redox-sensitive transcription factors triggers the production of proinflammatory cytokines, which regulate inflammatory responses such as macrophage activation, nitric oxide production, and smooth muscle cell proliferation.

The most studied cytokines are tumor necrosis factor (TNF), IL-6, and interleukin-8 (IL-8), which are regulated by the activation of NF-κB and AP-1 (Lavie and Lavie 2006; Panes and Granger 1998; Haddad and Harb 2005). Elevated levels of proinflammatory cytokines, adipokines, and adhesion molecules also show activation and acquired prothrombotic phenotype in blood cells and endothelial cells of patients with OSA (Lavie 2009).

23.4.2 Diagnosis and Treatment of OSAS

Studies have shown that sleep apnea increases atherogenic events. This process seems to start at the onset of sleep apnea syndrome. Although the patients referred to clinics after developing the major symptoms of OSAS tend to be in the fifth decade of life, Wisconsin studies revealed that of men in the third decade of life, 17 % have mild and 6.2 % have moderate sleep apnea (Young et al. 1993). People with disordered breathing are likely to develop hypertension within 4 years (Lavie and Lavie 2006). These findings show that the cardiovascular damage in OSAS is progressive and accumulates over time. When the diagnosis is late, the risks of mortality and morbidity are higher (Lavie and Lavie 2006).

23.5 Treatment of OSAS and Its Effects on Oxidative Stress

23.5.1 Continuous Positive Airway Pressure

CPAP therapy is the preferred treatment option for patients with OSAS. CPAP treatment is usually delivered via a nasal mask and helps to maintain upper airway patency, so it is a treatment, not a cure for the disease, and CPAP treatment has many benefits, such as decreasing daytime sleepiness, improving neurocognitive function, and reducing cardiovascular disease (Friedman et al. 2012). Barcelo et al. (2006) studied the effects of CPAP treatment on oxidative stress in patients with OSAS and found that continuous CPAP treatment improves the antioxidant defense. As oxidative stress and endothelial dysfunction are important predictors of an increased risk of cardiovascular disease, this might explain the decrease in cardiovascular disorders after CPAP treatment (Barcelo et al. 2006). Tothova et al. demonstrated that evening concentrations of the salivary thiobarbituric acid-reacting substances ($p<0.001$), advanced glycation end products ($p<0.001$), and advanced oxidation protein products ($p<0.01$) were significantly lower than morning values during the diagnostic night. However, they found that salivary concentrations of none of the oxidative stress markers were significantly influenced by the CPAP treatment. No changes in salivary antioxidant status after CPAP therapy were found (Tothova et al. 2013).

23.5.2 Antioxidant Treatment and OSAS

Sadasivam et al. (2011) suggested that oral intake of the antioxidant *N*-acetylcysteine improves sleep parameters and produces beneficial effects on oxygen saturation.

23.5.3 Surgical Treatment and Oxidative Stress

Skelly et al. (2013) studied the oxidative stress in upper airway muscles; they found that intermittent hypoxia and hypoxia reoxygenation have an equivalent negative inotropic effect on isolated rat sternohyoid muscle force. A superoxide scavenger TEMPOL increases sternohyoid muscle sensitivity to electrical stimulation and was modestly effective in preventing muscle weakness, but, however, failed to recover decreased upper airway muscle performance during sustained hypoxia. Akpinar et al. (2012) studied on the salivary myeloperoxidase levels in the saliva of the OSAS patients and normal controls; they found that salivary myeloperoxidase was higher in OSAS patients which may show the local inflammation. According to these studies, it can be supposed that OSAS is both a systemic and local oxidative stress disorder.

Lee et al. (2009) studied on the effects of uvulopalatopharyngoplasty (UPPP) on serum levels of nitric oxide derivatives (NOx) and endothelial function by endothelium-dependent flow-mediated dilation (FMD) in OSAS. They found that success of the procedure was correlated with renormalization of NOx levels and FMD. These results are consistent with the measurement of NOx levels in patients whose OSAS was successfully treated with CPAP.

Gozal et al. (2007c) have shown that nonobese children with OSA are at risk for endothelial dysfunction which has a correlation with circulating levels of sCD40L in these children. The marker for endothelium-related activation and dysfunction is soluble CD40 ligand (sCD40L), which binds CD40 on the surface of various cell types; such as endothelial cells, and triggers the increment in the expression of inflammatory mediators, growth factors, and the procoagulant tissue factor. They concluded that the effective treatment of OSA by adenotonsillectomy may not be associated with reversibility of the functional endothelial deficits.

23.6 Genetic Studies

The studies of genetic alterations in OSAS reveal that the expression of specific genes is responsible for hypoxia-dependent ROS, resulting in physiological and sometimes pathological consequences. The expression of these genes depends on redox-sensitive processes. Some of the genes studied are HIF-1, NF-κB, AP-1, early growth response-1 (EGR-1), nuclear factor-interleukin-6 (NF-IL6), and Sp-1 transcription factor (Sp-1) (Lavie 2003).

NF-κB and AP-1: NF-κB is needed for the expression of TNF-α and IL-1, chemokines, growth factors, and adhesion molecules, which have an importance in inflammatory responses and atherosclerosis (Lavie 2003). NF-κB initiates inflammatory pathways and regulates the production of adhesion molecules, inflammatory cytokines, and adipokines. Moreover, NF-κB is also associated with obesity and the metabolic syndrome, and in both conditions, induces inflammatory and atherosclerotic sequelae, (Lavie 2009).

NADPH Oxidases: NADPH oxidases are the main source of ROS in the vascular system. Several polymorphisms related to NADPH oxidase expression or activity have been identified. Pierola et al. (2011) compared the distribution of the allelic frequencies of A-930G and C242T polymorphisms in patients with OSAS and in a control group without OSAS. They found that the A-930G polymorphism of the p22phox gene may have an important role in genetic susceptibility to OSAS and the C242T and A-930G polymorphisms of the p22phox gene may be involved in the development of oxidative stress in OSAS patients.

HIF-1: HIF-1 activates transcription of genes responsible for adaptive responses to reduced O_2 by the carotid body. HIF-1 is a global regulator of oxygen homeostasis that controls multiple key developmental and physiological processes including angiogenesis and erythropoiesis (Semenza and Prabhakar 2007).

TNF-α gene is associated with sleep latency reduction and excessive daytime sleepiness. The proinflammatory cytokines TNF-α, interleukin (IL)-6, and IL-1α were more highly expressed in the OSA-derived tonsils of children (Tan et al. 2013).

23.7 Conclusion

OSAS is a systemic disorder that affects the cardiovascular and neurocognitive systems. Patients with OSAS have increased oxidative stress levels and reduced antioxidant enzyme activities. Increased oxidative stress in OSAS patients may explain some of the associations among OSAS, hypoxia, and the risk of cardiovascular disease in OSAS patients. Further studies about oxidative stress and its genetic etiology are needed to define the role of oxidative stress in these associations in OSAS.

Systemic inflammation is another aspect of pathological mechanisms for the consequences of OSAS. There is usually a coexistence with cardiovascular disease, type 2 diabetes, asthma, and smoking, all of which have effects on systemic inflammation and oxidative stress.

Future studies should deal with the confounding effect of obesity and the coexistence of other conditions that affect the same mechanisms as OSAS does. Most studies indicate that oxidative stress increases in OSAS and that it can be partially improved by CPAP treatment. However, there are still conflicts about the biomarkers of oxidative stress and effects of surgical treatment (Arnardottir et al. 2009).

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Chapter 24
Free Radicals in Nasal and Paranasal Diseases

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Abbreviations

AGE	Advanced glycation end-products
AMP	Adenosine monophosphate
AOPP	Advanced oxidation protein products
ATP	Adenosine triphosphate
GPx	Glutathione peroxidase
GRX-1	Glutaredoxin, glutathione-dependent oxidoreductase 1
GSH	Reduced glutathione
GSSG	Oxidated glutathione
IgE	Immunoglobulin E
IL	Interleukine
INFγ	Interferon γ
iNOS	Inducible nitric oxide synthase
NO	Nitric oxide
oxLDL	Oxidated low density lipoprotein
PGE	Prostaglandin

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RAGE	Receptor of advanced glycation products
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TNFα	Tumor necrosis factor-α

24.1 Introduction

Diseases of the nose and paranasal sinuses are most often of allergic, infectious (viral, bacterial, or mycotic) or neoplastic origin. In addition to the nasal cavity itself, the maxillary and ethmoidal sinuses also frequently are involved.

Complications of nasal and paranasal diseases involving structures other than the orbit are comparatively rare. A subperiosteal abscess of the scalp, also known as Pott's Puffy Tumor can result from the spread of infections from the frontal sinuses.

Tumors of paranasal sinuses are very prone to invade the orbit. Malignant tumors of paranasal sinuses erode the walls of the orbits and often present as orbit-related complaints. A benign or malignant tumor might present as proptosis and diplopia. Malignancies can infiltrate the oral cavity and/or nasopharynx as well. Another feature can be snoring, which can be caused by nasal obstruction from benign or malignant tumors. Many diseases are linked to damage caused by reactive oxygen species (ROS). An imbalance is called oxidative stress and occurs if there are increased levels of ROS and/or reduced levels of antioxidants.

24.2 Free Radicals and Their Action on Nasal and Paranasal Sinus Tissues

Free radicals participate in many diseases and states, e.g., inflammation, tumors, atherosclerosis, degenerative neurological diseases, etc.

Free radicals in the nasal cavity can be produced in many ways. Free radicals mostly come from white blood cells, which release them as part of the inflammatory response. Increased amounts of free radicals released by leucocytes during inflammation can kill bacterial, molds, yeasts, and parasites. Formation of free radicals is supported by cytokines IL-1 (interleukin-1) and TNF-α (tumor necrosis factor-α). Other sources of free radicals are reperfusion after ischemia and cell necrosis associated with the release of purines which are oxidized by xanthine oxidase to produce two superoxide radicals. Free-radical formation occurs in association with bleeding into tissues which releases iron that catalyzes the Fenton's reaction and generates free hydroxyl radicals from hydrogen peroxide. In mucous tissue, free radicals can be formed by exposure to UV light. Mucous membranes lack melanin and therefore lack UV protection. Exogenous free radicals can come from air pollution, from foods, and from cigarette smoke. Hyperglycemia is also a source of free radicals via AGE-substances (advanced glycation end-products), which can destroy proteins and

upregulate catalase other generators of free radicals. Formation of these free radicals can be blocked by amino-guanidine, lysine, nicotinamide, thiamine pyrophosphate, pyridoxamine, and certain antioxidants.

Free radicals can be created in association with reperfusion after ischemia, during ischemia via metabolism of purines released from DNA of damaged cells, catecholamines, prostaglandins, advanced glycation end-products, during biosyntheses of uric acid, in diabetes mellitus, during renal insufficiency, and from external sources like X-rays, harmful air pollutants, UV light, various drugs, tobacco smoke, psychological stress, pain, and many other factors. Each puff of a cigarette contains 100 trillion free radicals of varying types. Hypochlorous acid from hydrogen peroxide is formed as a result of the action of myeloperoxidase in nasal tissues. This acid harms tissues; however, in low concentrations hypochlorous acid (HOCl) has been shown to exhibit both antibacterial and anti-influenza virus activity. HOCl treatment can significantly inhibit human rhinovirus-induced secretion of IL-6 and IL-8 and significantly reduce viral titer (Yu et al. 2011). Some of the most common ROS are superoxides, free hydroxyl radicals, singlet oxygen ($^1O_2$), hydrogen peroxide, peroxynitrite ($ONOO^-$), and nitric oxide (NO). Ozone directly increases the level of free radicals and DNA synthesis. Exposure to ozone impairs mucociliary transport by nasal epithelia, increases cell permeability and facilitates the influx of inflammatory cells with proliferative and secretory responses. Cytokines are released, as well as cyclooxygenases and lipoxygenases which increase free radicals and decrease mucociliary clearance.

24.3 Antioxidants Operating in Nasal and Paranasal Sinus Functions

Due to the number of free radicals and ROS involved in nose pathology, antioxidants are important to maintain normal function or correct dysfunction. There are many antioxidants produced by the body, or absorbed from food or even consumed directly as drugs or chemical compounds. Intracellular antioxidants include reduced glutathione and thioredoxin reductase, while extracellular antioxidants include uric acid, albumin, proteins, bilirubin, vitamin C, β-carotene, vitamin E, and folic acid. Enzymatic antioxidants include superoxide dismutase, glutathione peroxidase, glutathione reductase, and catalase, while inorganic antioxidants include selenium, zinc, and magnesium. Antioxidant drugs include allopurinol, local anaesthetics, calcium channels blockers as well as many others. Quercetin and coenzyme Q_{10} are locally acting antioxidants that can protect mucosal cells of the nasal turbinates which have previously been in contact with hydrogen peroxide (Reiter et al. 2009). Coenzyme Q_{10} inhibits mitochondrial lipoperoxidation, supports ATP (sodium adenosine triphosphate) production and ROS removal. Therapeutic application of coenzyme Q_{10} is limited it poor solubility and poor bioavailability (Fetoni et al. 2009). Physical training increases total antioxidant capacity, uric acid, SOD and GPx and decreases Complex Regional Pain Syndrom - Type - I. (Rahman et al. 2010; Gonzáles et al. 2008).

Unpaired electrons are needed to destroy free radicals. The earth has a limitless supply of free and mobile electrons. Molecular hydrogen (H_2) acts as an antioxidant and removes free hydroxyl radicals ($^{\bullet}OH$), improves sleep, and decreases pain and inflammation. Molecular hydrogen has the ability to rapidly diffuse across membranes, it can reach and react with cytotoxic ROS and thus protect against oxidative damage.

24.4 Inflammations

Inflammation is a common feature of nasal and paranasal diseases. Inflammation can be caused by free radicals, which in turn attract leucocytes to the inflamed area, which then increase production and release of free radicals in an effort to fight inflammation. Inflammation, infection, and sepsis also attract increasing numbers of phagocytes, especially macrophages. Dead and dying leucocytes, in the form of pus, represent a source of free radicals that must be removed to avoid further free radical-induced damage. This is especially important in newborns, since their antioxidant defense system is immature and undeveloped. Inflammation triggers production of free radicals in phagocytes and target cells through TNFα. This initiates an inflammatory cascade in which free radicals act on macrophages further activating TNFα, and which in turn leads to the release of IL-1, IL-2, IL-6, IL-8, and IL-12. This cascade leads to an increase in hydrogen peroxide, the additional release of cytokines, and the formation of prostaglandin (PGE2), leukotrienes, and other inflammatory mediators. Activation of thrombocytes causes release of arachidonic acid from platelet cell membranes. Arachidonic acid is a precursor for prostaglandins, leukotrienes as well as the vasoconstrictive substance thromboxane A2. Compounds which protect the integrity of proteins are blocked by free radicals, which leads to an increase in elastase, collagenase, and other compounds that degrade proteins. Oxidative stress causes a decline in immunity. The free radical superoxide lowers the level of antibodies produced in response to immunization. Glycated proteins, AGEs and peptides are receptor agonists (RAGE—receptor of advanced glycation end-products) and lead to long-lasting inflammation, followed by chronic production of new free radicals. Inflammation and markers of inflammation can be reduced through the use of antioxidants. Antioxidants used to treat inflammation can improve immunity and diminish the actual inflammation itself. Antioxidant therapy is useful for treating oxidative stress, but the interrelationships between free radicals, cytokines, and activated lymphocytes are very complicated and it cannot be just assumed that oxidative stress causes immunodepression and antioxidant therapy leads to immunostimulation.

In chronic inflammation and uveitis, low serum levels of zinc and selenium are often found. The level of these trace elements tends to decrease with age, tending to make older individuals more susceptible to these inflammatory conditions.

24.5 Sinusitis

Inappropriate expression of genes that maintain the sinonasal innate immune system very probably explains the pathogenesis of chronic rhinosinusitis with nasal polyps. Activated eosinophils may lead to the production of hypobromous acid (HOBr) and hypochlorous acid (HOCl), which are modified to 5-bromocytosine and 5-chlorocytosine. Then aberrant methylation of cytosine, during DNA replication, leads to an alteration in gene expression seen in patients with chronic rhinosinusitis with nasal polyps (Seiberling et al. 2012).

There are various relationships between bronchiectasis, chronic rhinosinusitis, and nasal polyposis. Most patients with bronchiectasis also have rhinosinusitis with rhinorrhea and nasal congestion. Patients with chronic rhinosinusitis have lower levels of NO before, as well as after, the operation. Patients with polyposis have significantly lower nasal NO levels (Guilemany et al. 2009). Olfactory function and nasal NO concentrations are correlated in chronic rhinosinusitis patients but not in healthy subjects. The production of nasal NO by paranasal sinuses does not seem to directly influence olfactory function (Elsherif et al. 2007). Exhaled NO appears to come mainly from the paranasal sinuses and nasal mucosa. Metabolites of NO and NO itself are significantly higher in the maxillary sinuses of patients with chronic sinusitis. Increased levels of NO and its metabolites in the sinuses are correlated with damage to the epithelia lining healthy sinuses (Naraghi et al. 2007). Olfactory function and nasal NO are both reduced in patients with chronic inflammatory sinonasal diseases, but without a clear explanation of its cause. Nasal NO production seems to decrease with age and is associated with overall olfactory function (Gupta et al. 2013).

Exhaled NO is produced by the respiratory mucous membranes of the nose and accessory nose cavities. Levels of NO metabolites are significantly higher in the maxillary sinuses, especially in patients with chronic sinusitis. The impaired epithelia lining of the sinuses play an important part in the pathogenesis of sinusitis (Naraghi et al. 2007). In acute maxillary sinusitis, increased lipoperoxidation (increased malondialdehyde) and SOD are seen in the mucosa. In chronic sinusitis there are decreased levels of serum IL-12, alpha-tocopherol, uric acid, and SOD found in the tissues.

The lower the levels of GSH (reduced glutathione), uric acid and SOD, the more severe the disease appears to be. Antioxidant supplementation may be helpful. Biopsies of the nasal mucosa have shown decreased level of GSH, uric acid, and decreased antioxidant capacity in patients with chronic sinusitis. There were no significant differences with regard to vitamin E and GSSG (oxidized glutathione) levels relative to controls. GSH is consumed as it removes free radicals; a decrease in uric acid levels can be explained by the protection from oxidation by vitamin C and the binding of transition metals. This reaction irreversibly degraded uric acid, which is not synthesized in the nose, but is absorbed there from blood. The air along with NO is humidified during passage through the nasal sinuses. This increases the power of the airways to defend against radicals (Akatov et al. 2000; Westerveld et al. 1997).

24.6 The Influence of Nitric Oxide on Nasal and Paranasal Tissues

Nitric oxide is a diffusible, transient, reactive molecule that has physiological effects in the picomolar-to-micromolar range. Acting through soluble guanylate cyclase activation, NO is an important physiological regulator of the cardiovascular, nervous, and immunological systems. NO is bioavailable through two routes. It can be endogenously generated by constitutive or induced enzymes like nitric oxide synthase. The formation of NO from L-arginine represents an alternative pathway for nitric oxide generation. Nitrates and nitrites can be reduced to nitric oxide and other bioactive nitrogen oxide species, especially during hypoxia and acidosis. Orally ingested nitrates/nitrites are rapidly taken up into the circulation and subsequently converted to NO-species. This contributes to the defense of the tissues, regulation of blood flow, cell metabolism, and NO signaling. NO has multiple effects on tissues. NO plays an important role in vasodilatation, bacterial aggression, cytokine stimulation, regulation of mineralized tissue function, neurotransmission, platelet aggregation, etc. However, under pathological conditions, it can be quite damaging as in periodontal disease and oral cancer (Udgar-Cankal and Ozmeric 2006; Weitzberg et al. 2010). Chewing supports its production and increased perfusion accelerates healing. Nitric acid and saliva radical have significant antibacterial effects. The same antimicrobial effect has saliva (Rettori et al. 2000). Smokers have lower levels of NO than nonsmokers (Hays et al. 1992; Seri et al. 1999). Decreased serum levels of NO and increased levels oxLDL (oxidized low density lipoproteins) are also seen in patients with obstructive sleep apnea-hypopnea syndrome. This can promote the formation of atherosclerosis. There is a correlation between endothelial dysfunction and the severity of hypoxia (Jiang et al. 2011; Marteus et al. 2005).

Cigarette smoking reduces the level of nitric oxide in exhaled air. This reduction occurs mainly in the oropharynx. Smokers exhaled 67 % lower levels of NO than controls. There were no significant differences in salivary nitrite, nitrate or ascorbate between smokers and nonsmokers. Cigarette smoking does not affect nonenzymatic NO formation from nitrite in saliva, but decreases enzymatically produced NO (Marteus et al. 2005). Mouthwash with antibacterial chlorhexidine reduced salivary nitrite by 65 % and exhaled NO levels by 10 %.

24.7 The Diseases of the Nose and Sinuses

The nitric oxide radical (NO) is very important in these diseases. NO is a fundamental signal molecule associated with wound healing. It is an important cofactor with regard to the migration and proliferation of keratocytes as they relate to angiogenesis and collagen deposition. The level of NO in the nose is affected by age, physical load, smoking, and a variety of drugs. Vasoconstrictive drugs decrease the level of NO in the nose, but the reduction can be due to a mechanism other than vasoconstriction. Vasoconstriction of mucous membranes in nose is affected by the physical

load, but NO is not reduced under this condition (Serrano et al. 2007). Neuropeptide Y reduces the passage of blood through the nasal mucous membranes. Obstruction of air flow through the nose is influenced by thromboxane A_2 which causes congestion and leukotriene D_4 which likely produces vessel dilatation in the nasal mucosa. Pathological levels of NO are primarily associated with ciliary dyskinesis, allergic coryza, sinusitis, nasal polyposis, and cystic fibrosis. NO functions are mainly protective i.e., antimicrobial and antiviral. Antibiotics in sinusitis will not prevent free radical impairment in the tissues. Addition of vitamin A to the antibiotic treatment regime decreases lipoperoxidation and reduces the extent of histopathology (Guven et al. 2007). However, SOD activity was found to be higher when antibiotic therapy was used alone. Bioparox spray inhibits the synthesis of free radicals and the effects of IL-1 and TNF-α; while at the same time it potentiates the anti-inflammatory effects of IL-2 and INF-γ (interferon-γ).

During a nose bleed large amounts of superoxide radicals are released, which raises the activity SOD and reduces the levels of NO in expired air. Hypertension and impaired vessels in the nose are predisposing factors for nose bleeds.

NO protects against intranasal infection by viruses, e.g., type-1 herpes simplex. Blockade of nitric oxide synthase by amino-guanidine increases inflammation. NO causes not only vasodilatation, through smooth-muscle relaxation, but also acts as a neuromediator. NO is produced mainly in the nasal cavity and in the paranasal sinuses. The amount of expired NO in women is 25 % lower than in men. Smoking causes a decrease of 50 % (Taylor et al. 2007). NO is also lower in chronic maxillary sinusitis, although high concentrations are associated with allergic rhinitis, polyposis of the nose, and infections of the upper respiratory system. In inflammatory sinusitis, the mucosa has higher SOD activity, but lower GPx activity. The reaction of SOD with superoxide forms peroxynitrite, which reacts with tyrosine to form 3-nitrotyrosine. This compound is found in the respiratory epithelial tissue of patients suffering from vasomotor rhinitis.

24.7.1 Allergic Rhinitis

Allergic reactions require two signals: (1) the presence of the allergen and (2) ROS. Additionally, the production of hydrogen peroxide by eosinophils is an important component in tissues damage as well as a facilitator of allergic reactions. Mast cells release some inflammatory mediators that contribute to the pathogenesis of several diseases, e.g., allergic rhinitis. Tissue damage due to mast cells can be ameliorated with vitamin E. Children with allergic asthma have higher amounts of expired NO than the children with allergic rhinitis. Decreased levels of NO are found in those with a chronic cough and after exposure to tobacco and alcohol (Djupesland et al. 2001). Particulate matter in polluted air (caused by petroleum engines) can cause allergic rhinitis and even asthma (Wan and Diaz-Sanchez 2007); in these cases, an increase in antioxidant enzymes appears to be chemoprotective (Marteus et al. 2005). There is a positive correlation between NO and IgE

(immunoglobulin E) in their serum. High levels of IgE in the serum are a specific and predictable marker to differentiate asthma from allergic rhinitis. The function of smell and the concentration NO in the nose is positively correlated in patients with chronic rhinosinusitis, but not in healthy people. Smell is impaired in Alzheimer patients due to oxidative stress (Getchell et al. 2003). In cases of primary ciliary dyskinesis, in children, the level of NO is low. In contrast, levels of NO in the expired air of newborns with chronic pulmonary disease are higher than in the exhaled air of healthy subjects (Baraldi et al. 2004). Expired nitric oxide is a noninvasive marker of airway inflammation in asthma. Allergic rhinitis, asthma, and glucocorticoids all increase levels of nitric oxide. Vitamin E may decrease the formation of nitric oxide, but the effects of vitamin E were not confirmed to be effective during administration associated with five common allergens.

If the level of NO from the upper respiratory system is low, during a screening test, then it suggests that ciliary dyskinesis can be excluded from the differential diagnosis (Corbelli and Hammer 2007).

Allergens that are associated with pollution contain NADPH-oxidase which generates ROS. These substances then play a role in pathogenesis of allergic inflammation that originates in the airways.

Nitric oxide inhibits epithelial expression of several proinflammatory cytokines and can inhibit viral replication caused by the rhinoviruses. Increased production of nitric oxide evokes fewer symptoms of disease and accelerates the removal of viruses (Proud 2005).

In obstructive sleep apnea there is a lower concentration of NO in alveoli and the upper respiratory airways. Expired air from lungs has a lower pH in obese patients regardless of whether they have or do not have obstructive sleep apnea (exhaled acidopnea). Apnea increases the oxidative stress and effects cardiovascular morbidity. In the morning, patients have increased lipoperoxidation and decreased amounts of the antioxidant enzyme, paraoxonase-1 with continual positive air pressure air flow, there is no decline in NO levels. Continual positive air pressure air flow ameliorates the syndrome of sleep apnea. Obstructive sleep apnea is often combined with inflammation of nasal cavity, with congestion of the mucous around the uvea and hyperreactive airways. Free radicals are the main cause of these problems, especially when they are linked to postischemic reperfusion.

Treatment of persistent allergic rhinitis with levocetirizine improved nasal inflammation, rhinorrhea, nasal itching, and air flow patency; additionally, there was a transient improvement in the sense of smell (Guilemany et al. 2012). AOPP (advanced oxidation protein products) levels were significantly higher in the blood of patients with allergic rhinitis than in controls (Aksoy et al. 2009). The level of serum NO, the ratio of inducible NOS to total NOS, and the activity of GPx in patients with allergic rhinitis were significantly higher than those seen in the healthy control group. However, the levels of malondialdehyde and SOD activity were not statistically different compared to the control group (Jiao et al. 2010). The nasal fraction of exhaled NO was higher than the oral fraction of NO.

In patients with chronic rhinosinusitis, the oral fraction of NO was higher than in controls, but when seen in combination with nasal polyps, the nasal NO fraction

was lower. So differentiating hyperplastic eosinophil rhinosinusitis from chronic nonspecific rhinosinusitis is possible as in effective monitoring of the clinical course of chronic rhinosinusitis with polyps (Weschta et al. 2008).

24.7.2 Polyps

The pathogenesis of nasal polyposis is multifactorial. The high levels of malondialdehyde in polyp tissue reflect the participation of free radicals. Nose polyps are considered to be a response of tissues in an inflammatory state associated with oxidative stress and increased counts of inflammatory cells, especially eosinophiles. Nasal epithelium plays a critical role in the formation of polyps. The level of NO in the nose of patients with nose polyps is lower than that of patients with uncomplicated allergic rhinitis. An increase in NO lowers the volume of polyps. A combination of oral and nasal measurements of fractional concentrations of exhaled nitric oxide is useful for monitoring the extent of inflammation in patients with polyps and eosinophilic chronic rhinosinusitis. In a surgical therapy group, the increase in the fractional concentration of exhaled nitric oxide indicated a prompt recovery of NO released from the sinus mucosa (Noda et al. 2012). Nose polyps and asthma are related disorders. In nose polyps there is decreased activity of GPx, while the activity of catalase and xanthine oxidase was increased; malondialdehyde was also increased and serves as a sign of free radical damage. The amount of free radicals in polyp tissue corresponds to the severity of the disease (Cheng et al. 2006). Myofibroblasts in nasal polyps are involved in nasal polyp formation by inducing extracellular matrix accumulation. ROS are released during the differentiation of fibroblasts to myofibroblasts. Stimulation with transforming growth factor-β1 increased ROS production by nasal-derived fibroblasts. Myofibroblast differentiation and the production of collagen in nasal polyp-derived fibroblasts can be prevented by inhibition of ROS with diphenyliodonium, *N*-acetylcysteine, and ebselen (Park et al. 2012). AOPP can be used as a marker of oxidative stress in the etiology of nasal polyps. Patients with nasal polyps have significantly higher levels of AOPP than subjects without polyps (Veyseller et al. 2010). Inflammatory conditions in nasal and paranasal sinus cavities are evidently suitable relative to the formation of nasal polyps. There is a strong relationship between oxidative stress and the pathogenesis of nasal polyps (Cekin et al. 2009). Expression of GRX-1 (glutaredoxins, glutathione-dependent oxidoreductase) in nasal polyposis is significantly higher than in normal nasal mucosa. The expression of GRX-1 is evidently the primary defense against chronic inflammatory oxidative stress of the nasal mucosa. IL-1β increases the production of intracellular ROS. This reaction is inhibited by *N*-acetylcysteine. Glucocorticoids can regulate the expression of GRX-1 and IL-1β-induced ROS formation (Woo et al. 2009). Peroral and intranasal application of steroids ameliorates nasal airflow patency; acoustic rhinometry and nasal NO are suitable methods for monitoring and followup of patients with nasal polyps. Intranasal application of steroids in cases of nasal polyps ameliorates nasal airflow

patency and paradoxically increases the nasal levels of NO (Alobid et al. 2012). Inhaled AMP (adenosine monophosphate) narrows air flow passages and reduces air flow more in patients with nose polyps than in controls. Oxidative stress is an important feature of nasal polyp and asthma pathophysiology. There is a causal relation between oxidative stress and numbers of inflammatory cells, especially eosinophils. There is a positive correlation between the severity of nasal polyposis and free radical levels (Cheng et al. 2006). Nasal polyps are considered to be an inflammatory condition of the nasal and paranasal sinus cavities with an unknown etiology. Malondialdehyde as a major end-product of lipid peroxidation and superoxide dismutase and nitric oxide as antioxidants play important roles in oxidative stress. The concentration of malondialdehyde has been shown to be significantly higher, and superoxide dismutase and nitric oxide levels are significantly lower in patients with nasal polyps compared with a control group. This demonstrates that there is a strong relationship between oxidative stress and the pathogenesis of nasal polyps.

24.7.3 Tumors

Oxidative stress is initiated by an abundant formation of free radicals and/or due to a decrease in the total antioxidant defenses of an organism. Oxidative stress significantly contributes to the origin of tumors, evokes DNA mutation, and destroys macromolecules and tissues. Repetitive and cumulative damage to cellular organelles caused by free radicals leads to 3× higher values of malondialdehyde (Dwivedi et al. 2008). The growth of tumors is dependent on angiogenesis, which is supported by activators of angiogenesis and by hypoxia. Some antioxidants slow angiogenesis. Without angiogenesis a tumor can only reach a size of about 1 mm^3. Normally there is a balance between activators of angiogenesis and angiogenesis inhibitors. The presence of activators, together with hypoxia can initiate angiogenesis and tumor growth. Hypoxia actually has a selective effect and tends to select for the most malignant cell mutants. Hypoxia effects local acidosis; waste products from tumor cells are transported into the lymph and to the surrounding interstitium, where they increase the osmotic pressure and create a milieu that makes drug penetration difficult. The tumor microenvironment, which tends to be more alkaline, contributes to the formation of resistance to chemotherapeutic drugs. Tumor cells may first appear as a reaction to hypoxia. Hypoxia increases the level of angiogenic growth factors and increases the associated gene expression, which supports tumor growth. Hypoxic conditions support the survival of tumor cells, which are more aggressive and less susceptible to hypoxia (Dye and Adler 1994).

Free radicals can promote DNA mutations, lipoperoxidation, which is accompanied by increased production of carcinogenic aldehydes. Solid tumors neither activate nor inhibit NO effects (Selimoglu 2005). High concentrations of nitric oxide radicals accelerate tumorigenesis, oxidative stress in obesity and aging, chronic infections, smoking, etc. and increase the incidence of carcinomas. In contrast, the presence of antioxidants like selenium, melatonin, resveratrol, glutathione, bioflavonoids, etc. has been shown in many cases to decrease tumor proliferation.

Free radicals are often found in advanced carcinomas, however, in these cases the administration of antioxidants could interfere with therapy. It is known that cancer cells absorb antioxidants more rapidly than healthy cells. This in turn protects them from free radical therapy.

There is no direct contact between cigarette smoke and oropharyngeal mucosa. Cigarette smoke contacts the saliva before it reaches the mucosa. Low reactive free radicals from smoke interact with redox-active metals in saliva and result in the production of highly active free radicals that decreases the antioxidant capacity of saliva. Therefore the aldehydes in cigarette smoke destroy the protective components in saliva, such as peroxides and antioxidant enzymes. In otopharyngeal cancer, saliva plays a pivotal role in the cancer pathogenesis. Chronic inhalation of cigarette smoke is associated with mucus hypersecretion, with mucus pooling, pulmonary connective tissue damage, and chronic airflow obstruction. Cigarette smoke has also been established as an important risk factor for nasal neoplasia. Cigarette smoke is responsible for 90 % of oral squamous-cell carcinomas, with free radicals and reactive nitrogen substances playing a very negative role, through rapid destruction of biological macromolecules like enzymes and proteins. Synergistic interactions of cigarette smoke, which contains free radicals, and saliva with redox-active metals decrease the antioxidant capacity, form a prooxidant state and support the formation of oral squamous-cell carcinoma (Nagler and Dayan 2006). Oral squamous-cell carcinoma is most often induced by exposure of oral epithelial cells to tobacco products. Cigarette smoke decreases lymphocytes survival times in the presence of saliva. Free radicals attract proteins with a concomitant accumulation of carbonyls. Proteins can be protected by addition of uric acid or glutathione, but not by ascorbate or *N*-acetyl-L-cysteine. Exposure of lymphocytes to cigarette smoke in the presence of saliva leads to a decreased lymphocyte survival rate; glutathione has been shown to be protective in this situation (Hasnis et al. 2004).

Nasal epithelium is the first respiratory epithelial surface exposed to environmental tobacco smoke. Cigarette smoke increases mucosal permeability and increases access of irritant or antigenic stimuli to epithelial nerve receptors. Cigarette smoke increases the number of free radicals in laryngeal and lung tissue, which in turn can lead to tissue environments conductive to carcinoma genesis. Vitamin E has been shown to decrease levels of ROS in these tissues (Uneri et al. 2006). Cigarette smoke can lead to the development of basal cell hyperplasia and squamous metaplasia in the nasal cavity; *N*-acetylcysteine and glutathione have been shown to be protective. Tracheal mucus from asymptomatic smokers has increased volume and water content compared to nonsmokers. Cigarette smoking has been associated with a slight elevation in the risk of squamous-cell carcinoma but not cancers of other cell types. Interaction between cigarette smoke and saliva may result in a rapid destruction of biological macromolecules such as enzymes and proteins, which can lead to oral squamous-cell carcinoma due to interactions between redox-active metals and reduced antioxidant capacity creating a prooxidant milieu (Nagler and Dayan 2006).

Malignant melanoma of the nasopharynx and paranasal sinuses is relatively rare. The findings suggest that melanoma of the nasopharynx and paranasal sinuses

contain wide ranging amounts of paramagnetic substances, both melanin and hemorrhage products.

Antioxidants decrease the level of free radicals and so can act preventively against the origin of malignant growth. Antioxidant supplements have negligible positive effects on healthy people, for example: preventing cancer or premature death. However, the majority of agents used to directly kill cancer cells, including ionizing radiation, most chemotherapeutic agents, and some targeted therapies, work directly or indirectly by generating ROS that block key steps in the cell cycle and kill cancer cells. Therefore, much of the difficulty in treating late stage cancer may be caused by the presence of too many antioxidants.

Dietary analyses have revealed significant protective effects associated with the consumption of garlic, oranges, and tangerines, with a 50 % reduction in the risk of nasal cancer among individuals in the highest intake groups of these foods. Consumption of salt-preserved vegetables, meat, and fish was associated with a significantly increased risk of nasal cancer in a dose-response fashion, with a five-fold increase observed in those with the largest intake of salt-preserved foods. These findings suggest that dietary factors may contribute to the development of nasal cancer. Decreased antioxidant capacity especially of glutathione and elevation of malondialdehyde in laryngeal cancer tissues cause lipoperoxidation. No significant differences were found between early and advanced tumor stages (Yilmaz et al. 2007). Oral cancer patients have lower levels of vitamins C and E resulting in a reduction in salivary antioxidant levels and increased lipoperoxidation (Rai et al. 2007). In laryngeal squamous-cell carcinoma, levels of ROS were seen to be related to the severity of the disease (Baglam et al. 2010).

Currently there are blood tests that detect antibodies to human papilloma virus (HPV), which can cause throat, oropharynx, hypopharynx/larynx cancers, and oral cancers, years before the symptoms of the disease become apparent. Seropositivity for HPV16 E6 has been found in prediagnostic samples from more than one-third of such patients.

A population-based case-control study of nasal cavity and sinus cancers, involving interviews of incident cases and controls, was conducted. Patients with a history of chronic nasal diseases, including conditions that occurred 10 or more years prior to the cancer diagnosis, were found to have a fourfold or higher risk of nasal cavity and sinus cancer compared to controls.

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Part X
Conclusion

Chapter 25
Overarching Challenges of Free Radicals in ENT Pathology

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25.1 Challenge One: Potential for Multiple Mechanisms

One of the overarching challenges in the prevention of acquired hearing loss is the fact that while many insults converge on free radical-based mechanisms of injury or disease, oxidative stress is not the only factor contributing to the pathological process. Free radicals are clearly important, but they are not the whole story; insults can induce pathological changes and dysfunction by other mechanisms as well.

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Clearly, there are also a variety of factors that can lead to an upregulation of excessive free radical formation, and this excess may lead to pathology and cell death via a number of downstream pathways; however, many of these factors may induce pathology via parallel pathways. While inflammation and apoptosis follow oxidative stress, it is unclear under what conditions these pathological processes proceed independently from oxidative stress. Associated immune reaction during stress and during the healing process initiates biochemical processes, some of which include free radical formation, which influence the degree of pathology induced. The extent of pathology can reflect a variety of exogenous and endogenous factors each differentially associated with each of these processes. Thus, it is not surprising that a focused antioxidant treatment that reduces oxidative stress may not provide complete prevention, particularly in the case of more robust insult to the ear or other head and neck tissues. While this should not delay translational studies, a better understanding of biochemical pathways involved in free radical-induced pathology in various cell types of the inner ear and how they interact, including hair cells, supporting cells, and cells in the lateral wall, as well as cell types of other structures of the head and neck affected by oxidative stress, will surely facilitate human trials allowing a more informed selection of putative interventions and more relevant measure of efficacy. A more nuanced understanding of the pathways to pathology, their interaction, and their hierarchy will guide the development of new "cocktails" of intervention, e.g., antioxidant, anti-inflammatory, and antiapoptotic agents targeting specific genes and signaling molecules, optimizing treatment of different types and levels of insult and disease.

25.2 Challenge Two: Choice of Animal Models and Rigor of Design

Current animal models may not be adequate to represent the human condition. Animal models are frequently good for a particular study of an insult and intervention; they are sometimes not good enough to provide needed generalization of result to a more complex human condition. Clearly we need tests across different animal models with different stresses, interventions, and dose response data on stress and intervention. Thus, a key issue is the selection of species, which may vary as a function of the disease and injury, the intervention, and the insult/injury. For example, the literature on otoprotection (against noise) has limited utility for comparisons across agents because the type and extent of the injury and the animal models vary considerably across the different assessed agents. With respect to hearing loss induced by drugs such as the aminoglycoside antibiotics and cisplatin, the injury induced in animal models often exceeds the injury in drug-treated patients. With respect to trauma induced by electrode insertion, it is difficult to precisely control mechanical injury in either animal models or human patients given that this varies with both surgical skill and patient anatomy. Moreover, the various factors, including the interplay of genetic and environmental factors, contributing to long-term slowly progressive pathology (evidenced as increasing hearing loss) are unclear.

These questions notwithstanding, this is a significant issue given that the majority of patients with residual hearing lose some or all postimplantation and with that lose some benefits.

In considering the utility of animal model data for design of human studies, it is increasingly clear that an "overarching" concern must include far greater rigor in animal investigations. Rigorous application of scientific methodology, including power analysis, blinding, carefully selected and time-distributed controls, objective and relevant outcome measures, careful adherence to statistical analysis including the identification in advance of primary measures for analysis, replication of results, and other factors must all be performed with care and thoughtful consideration (Landis et al. 2012; Katz et al. 2012a, b).

25.3 Challenge Three: Need for Relevant Metrics

Many of our current metrics do not span the animal model—human gap. As a field, there is a pressing need for metrics with general comparability between people and animals. There is a uniform agreement that conventional pure tone air conduction thresholds are a prerequisite starting point in humans, and auditory brainstem response thresholds are the typical correlate in animal subjects. However, the most common patient complaint is difficulty understanding speech in noisy or reverberant environments, and we need metrics in animals that can model these more complex perceptual deficits in humans. Two possibilities available are operant psychophysical tasks in which trained animals report discrimination of sounds with noise maskers in the background and collection of multiunit neural response data from inferior colliculus. While these measures are sufficiently time consuming and specialized that it is unlikely they can be incorporated broadly, selective use could bring great clarity to many important issues. One design paradigm that may be much more readily implemented within a broad variety of laboratories is the startle-based gap detection task, which can be a sensitive, albeit somewhat variable, measure of temporal processing, but which does not require animals be trained to perform an operant task. Frequency following response may provide a metric that could be adopted as a test for central auditory function. There is a broad agreement that human clinical studies should employ a test battery that includes a words-in-noise test; the National Institutes for Health (NIH) toolbox offers one possibility for a test that is optimized for speed and ease of use.

25.4 Challenge Four: Narrow Focus

A very focused and carefully controlled approach is frequently the most effective way to move basic science and our mechanistic understanding forward, and this approach can identify important factors that may play a role in human pathology. When defining therapeutic intervention in real-life situations, a broader view of the

factors and their interactions may be necessary to define an optimal strategy of treatment. Such a broader view, taking many factors into consideration, is complicated by increased effort and time and, with that, increased support required. The carefully controlled mechanistic animal laboratory investigation is expected to provide interpretable results. The carefully controlled human trial is hoped also to equally provide interpretable results, and it may, with data supporting an intervention with benefit. Unfortunately too often we now find that when the said intervention is applied to the appropriate population under far less controlled conditions, the benefits no longer "fit the intervention." One solution is more "real-life" test situations. These frequently offer less control, less interpretability, and with that less fundability. Another is epidemiological studies, with the weakness of less causality.

25.5 Challenge Five: Clinical Value and Societal Impact of Results

For translation of results from animal studies to human, we must better determine what can be considered a "clinically significant functional change." A small but reliable functional change after noise or drugs may be statistically significant without having any impact on subject performance in real-world conditions. Likewise, a drug might confer a small but statistically significant benefit, which has no impact on real-world deficits if there are robust and variable changes brought about by insult-related pathology. Power analysis related to clinically significant benefit should be incorporated in studies as part of the trial design process, with experienced biostatistical input. Consideration of pathologies of greatest impact on quality of life, productivity, and the number of individuals affected may condition our view of "clinically significant." Thus, a smaller effect on hearing impairment affecting many may be considered of greater societal value than a larger effect on fewer individuals. Thus, with the increasing life span, age-related hearing loss affects almost all of us, with profound effects on quality of life. Noise injury is one of the most common causes of acquired hearing loss in people around the world. Therapeutics that reduce or prevent noise-induced hearing loss potentially would benefit the largest numbers of patients, with significant effects on productivity and job satisfaction. Drug-induced hearing loss, following cisplatin or aminoglycosides, might have the biggest impact per patient given that the risk of hearing loss is great and the hearing loss so severe after many drug regimens. Aminoglycosides are certainly the largest cause of acquired hearing impairment in developing countries and second only to age and noise globally. Recent data indicating a relationship of hearing impairment and onset of cognitive dysfunction surely up the ante on the potential societal impact of preventing all hearing loss, particularly age-related. However, data supporting antioxidant benefit in the prevention of age-related hearing loss are limited and conflicting in animal models.

Obviously there are many other reasons that research priorities should take into consideration: pathology of greatest impact on quality of life, productivity, the number of individuals affected, and the impact on the economics of health care.

The reported interactions between hearing loss and cognitive deficits, and known relationships between hearing loss and depression, will likely generate significantly greater interest among scientists, physicians, patients, and pharmaceutical companies in prevention and treatment of hearing impairment. While all of the above are large, important populations, ISSNHL patients, along with cochlear implant patients, were identified as perhaps the most easily accessed patient population given multiple trials in the USA and in Europe in recent years. Prevalence, incidence, and severity are relatively well documented from these recent trials, and personnel at trial sites may be available to implement new trials with other agents. Making this difficult, the etiology of ISSNHL is usually unknown, and there is no adequate animal model for ISSNHL. Notwithstanding there are data indicating a role of oxidative stress in ISSNHL, and this untreatable disorder with its large impact on quality of life has been extensively studied for many other interventions for >50 years.

25.6 Challenge Six: Need for Epidemiological and Genomic Studies

There is a stunning lack of good epidemiological data exploring the relative contributions of the variety of factors that have been statistically associated with hearing loss and other head and neck diseases, many of which involve a free radical component. The disease processes that need to be identified include, but are not limited to, vascular and metabolic diseases that likely affect the ear and hearing and likely influence outcomes in head and neck cancer patients and patients with sleep apnea. A key obstacle is the inability to establish causal relationships between risk factors from epidemiological studies. Clearly epidemiological studies can be combined with prospective blinded trials to assess causation in "real-life" situations; but few of these have been performed in this field. Overlapping with this issue, there is a need for well-characterized patient populations with defined baselines and known risk for incidence and severity of pathological changes, such that a power analysis might be completed to optimize clinical trial design.

A significant major challenge with respect to individual risk is a better understanding of genetic risk factors. Genetic and epigenetic factors affect response to stress factors and efficacy of interventions and can mask secondary factors that complicate interpretation of results. Obviously these factors play back on the animal models we select and their application, or lack thereof, to humans. From genetic propensity to stress, treatment, immune disorders, and endogenous factors that modulate cancer to the length of the cochlea in implantation, these factors provide a different image depending on the genetic background on which it is viewed. While technology permits us greater access to assess and analyze greater numbers of potentially interesting genes, we are at a point of knowledge permitting us to narrow the field of known genes to a pool of the most compelling candidates. Proteonomic and genomic studies within the context of controlled human trials, in broad epidemiological studies or, perhaps best, in epidemiological studies with a prospective

blinded intervention assessment, will bring us closer to realizing personalized medicine. This knowledge will allow us to predict risk of pathology from various insults and potential interventions to prevent or treat and make them applicable for the individual in the reality of their lives.

25.7 Challenge Seven: Barriers to Performing Human Trials and Translation

Clearly problematic for all of the above is the issue of limited funding, an issue that is sometimes compounded (mentioned above) by a lack of appreciation by reviewers and funding agencies for some of the less exciting, but nonetheless necessary, steps in translational research for the development of drugs/technologies that will protect and treat the ear, nose, and/or throat. Dose–response data, for example, are time consuming, costly, and definitely not sexy and are too often perceived as having limited utility once a dose that "works" has been identified. However, to compare agents against each other and to effectively move from animal models to human trials, such data are essential. We need to be confident we are testing the best dose, not one ineffective or worse harmful, and using the optimal route of administration of any given agent or combination of agents. Also, as mentioned above, greater mechanistic knowledge, more appropriate animal models, and measures that readily translate between animals and humans are necessary.

On the human clinical trial side, we must begin to collect by default a variety of clinical data specifically including history of diseases, medications, and blood tests including lipid profile, hemoglobin, glucose, electrolytes, and kidney and liver function tests. In order to compare data from humans and animals, we should begin to collect health metrics in animals as well. We must find and characterize potential patient populations in which new drugs might be tested. Toward this end, the Department of Defense Hearing Center of Excellence is beginning an initiative to build a database on hearing loss as a function of specific military noise exposures. Complementary efforts are needed within specific organizations such as the Children's Oncology Group (COG), International Society of Paediatric Oncology (SIOP), and NRG Oncology, which bring together the National Surgical Adjuvant Breast and Bowel Project (NSABP), the Radiation Therapy Oncology Group (RTOG), and the Gynecologic Oncology Group (GOG). The goal is to ascertain populations available for multiple trials, similar to the trials at the clinical trials consortia at the National Cancer Institute (NCI). There is an acute and compelling need to build on collaborations between investigators in the United States and the European Union. To fully appreciate the potential benefits of these collaborations, new funding models that better support international collaboration are urgently needed. Building on the theme of international collaboration, new consortia that bring together experts to discuss ongoing activities and develop the next key steps will continue to advance critical but difficult translational research activity. One potential role for new consortia is to educate nonprofits about translational research, including the challenges,

the promise, and the cost. Targeted translational programs that bring together academic research resources with commercial enterprises with a clear mandate and pathway to treatment and market may well facilitate translational outcomes and may encourage nonprofits, industry, and venture capitalists to fund such programs, either in partnership with the government or independently. We recognize this latter proposal would require new conversations about intellectual property as universities and venture capital firms would have competing interests with respect to new patents, licensing, and royalties. But such issues have been resolved in the European Commission-funded programs, and clearly the benefits to the field and to human health and hearing outweigh the potential loss of partial revenue to both parties. To optimize the potential for funding from all possible sources, additional training for reviewers should be considered when applications are translational in nature, often yielding less mechanistic insight than the traditional investigator-initiated application. Funding agencies should continue to support calls for translational research applications.

25.8 Targets of Opportunity

While there are indeed many challenges to moving forward, there are also many targets of opportunity: There are many exciting and compelling areas of relevant research currently ongoing, some old and some new.

There are always new areas of special interest—one current example is the current great attention paid to synaptopathy (damage to synapses between the inner hair cell and auditory nerve). This raises the question of what is the role of free radicals in synaptopathy. Given the role of free radicals in excitotoxicity, we assume it may be significant. We must determine whether synaptopathy disrupts the processing of signals in noise or, more broadly, temporal processing, and if so, what is the critical boundary at which point functional deficits first emerge, and how do deficits grow with increasing synaptic loss. On this issue, speculation has far outstripped the available data.

Another target of opportunity is repurposing drugs. Drugs already approved by the FDA for other purposes in human patients should be tested for potential auditory system protection where possible in an effort to reduce some of the substantial costs associated with new drug development. For example, before the initial Phase I (safety) trials can be initiated in humans, the FDA will typically require pharmacokinetic data, toxicology, genotoxicity, and other safety data. This can be avoided if the information has already been acquired and approved for other types of studies and applications. The National Center for Advancing Translational Sciences (NCATS), a center within the NIH, has issued a funding opportunity announcement intended to facilitate the discovery of new therapeutic uses for existing molecules using new partnerships between industry and academia.

Another target of opportunity relevant to the development of new therapeutics to prevent hearing loss is the development of new smart drug delivery technologies. With respect to electrode insertion trauma, an electrode is the gateway to the

cochlea, opening doors to new methods for drug delivery via coated electrodes. More broadly, new miniaturized pumps that are smaller and more easily implanted and which have smart technology to deliver more than one drug, only when needed, may provide new local treatment options in the future. Local drug delivery will be essential if an otoprotectant disrupts antineoplastic or antibacterial effects of cisplatin or aminoglycosides, respectively.

A final target of opportunity is the development and application of personalized medicine. This can range from imaging a cochlea prior to surgery in order to choose the correct type and length of a cochlear prosthesis to taking into account a subject's genetic and epigenetic information as well as health history in designing therapeutics.

25.9 Conclusion

Exciting research in the past few decades has revealed a prominent role for oxidative stress in the pathology of the ear, nose, and throat (ENT). In the past decade, this research has built a substantial foundation of promising interventions that may prevent and treat many ENT pathologies. The chapters in this book, and the statement arising from this group of authors, provide novel insights and strategies to address pathology of the ENT that may be based on the generation of free radicals. Future research incorporating discussions held here promises to provide preventive measures to reduce morbidity from excessive free radical production and related mechanisms. Through this translational research process, patient quality of life will be maximized, and rehabilitation and disability costs to individuals, health-care organizations, and governments will be reduced. We envision a future where people do not have to lose their hearing, and pathology related to free radical formation can be broadly reduced in head and neck tissues.

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ERRATUM TO

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Acknowledgments page was missing in the original version of this book. The missing Acknowledgments reads as follows:

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