From Cochlear Cell Death Pathways To New Pharmacological Therapies

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Abstract: Ototoxic drugs, intense sound or age-related hearing loss recruit most of the apoptotic pathways, but also several additional enzymatic pathways which eventually result in cell death. Here, we discuss therapeutic strategies based on the inhibition of regulators or executors of apoptotic cell death pathways and on the use of antioxidants.

Key Words: Apoptosis, necrosis, cell death pathway, cochlea, therapeutic strategies, ototoxic drugs, intense sound, age-related hearing loss.

INTRODUCTION

In industrial countries, hearing impairment affects approximately 10% of the population and increases in frequency four-fold with aging. Age-related hearing loss (ARHL) or presbycusis is the major form of hearing loss, and the predominant neurodegenerative disease of aging [1].

Of all the sensory organs, the organ of Corti functions with the smallest number of sensory receptor cells: the auditory hair cells. The human cochlea only contains 15,000 sensory hair cells: 12,000 outer hair cells (OHCs) and 3,000 inner hair cells (IHCs). The OHCs, which have electromotile properties, feed energy into the mechanical system to enhance the vibration of a narrow region of the basilar membrane to improve the sensitivity and frequency selectivity of the organ of Corti. The IHCs are classical electromechanical transducers that convert sharp mechanical tuning of cochlear partition into a message that can be interpretated by the brain, i.e., action potentials conveyed by the auditory nerve to the central nervous system.

Hearing deficits are often caused by loss of sensory hair cells due to a variety of factors including ototoxic drugs (drugs causing damage to the auditory and the vestibular systems), intense sound exposure, infection, genetic mutations and aging. In mammals, auditory hair cells are produced only during embryonic development [2] and do not regenerate if lost during postnatal life, therefore a progressive loss of hair cells results in irreversible deafness. The concept that, in some instances, sensory hair cell death may be preventable has provided an exciting and novel direction for investigations aimed at the degenerative process. Signals that trigger sensory hair cell death can be as diverse as the removal of essential growth factors, damage by excitotoxins, exogenous toxins (including ototoxic drugs) and free radicals. An important facet to the newly discovered death cascade is that it can be halted, thus rescuing the dying sensory hair cell.

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This review outlines some of what is known about the mechanisms of sensory cell death due to ototoxic drugs such as aminoglycosides or cisplatin, intense sound exposure, or the aging process. The question of whether any of the sensory cell death observed after such insults occurs by an "active" programmed mechanism (apoptosis) is discussed. Innovative therapeutic strategies to prevent ototoxicity-, intense sound- or age-induced cochlear cell death are proposed.

1. NATURE OF CELL DEATH

Historically, the general mechanisms by which cells die have been classified into two general types, necrosis and apoptosis. Necrosis refers to the sum of degenerative changes that follow any type of cell death. Apoptosis is a tightly regulated, energy-dependent process in which cell death follows a programmed set of events. Currently, apoptosis has been classified into three types: apoptotic (type 1), autophagic (type 2) and non-lysosomal vesiculate or cytoplasmic (type 3) [3]. Typical examples of sensory hair cell death occurring in the cochlea are shown in Fig. **1**.

1.1. Apoptosis (Type 1)

Apoptosis is a well-documented active programmed cell death (PCD) in which the activation of caspases, a family of cell-suicide cysteine proteases, plays a central role [4, 5]. Morphologically, apoptosis is defined by cytoskeleton collapse, cell shrinkage, membrane blebbing, chromatin condensation, DNA fragmentation, loss of the asymmetry of phosphatidylserine (PS) in the plasma membrane and phagocytic elimination of the dying cell [6].

In the inner ear, moderate doses of ototoxins such as aminoglycosides or cisplatin have been shown to induce auditory sensory cell apoptosis *in vitro* [7- 9] and *in vivo* [10-12]. Several recent publications studying the biological effects of sound and impulse sound trauma revealed that apoptosis is the predominant mode of hair cell death following such sound trauma [13-15]. Raphael's group showed that the injured or dead hair cells and/or their debris following the intense sound or ototoxic drugs exposure are phagocytosed by the cochlear supporting cells within the epithelium [16].

The general mechanisms of cell death in aging remain to be elucidated. Recently, evidence has been accumulating that

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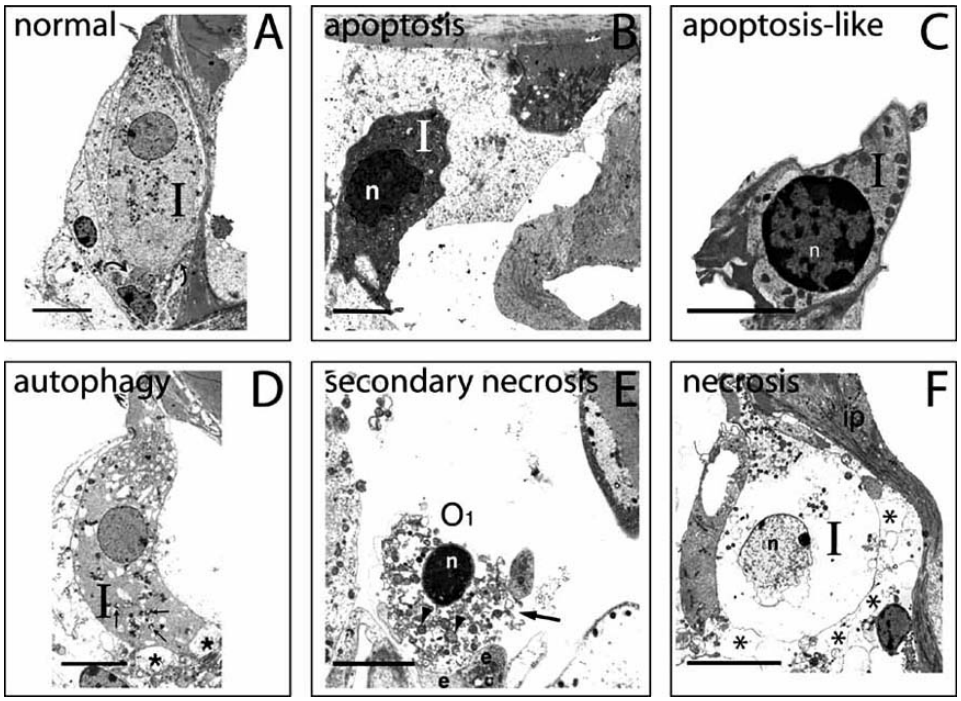

Fig. (1). Classification of cell death according to the nuclear morphology of the dying cell.

Shown are transmission electron micrographs. **A**: An IHC observed in an undamaged region of the organ of Corti 6 h after acoustic trauma. Both the IHC and its innervations (curved arrows) have a normal appearance. **B**: Damaged organ of Corti from a CDDP-treated animal. An IHC (I) showing characteristics of apoptosis, i.e., shrinkage of the cell body, and condensation of the cytoplasm with preservation of the cytoplasmic lateral membrane, with an electron-dense nucleus (n) due to chromatin compaction. **C**: A degenerating inner hair cell (I) from a neomycin-treated animal showing chromatin condensation that is less compact but more complex and irregular shapes, characteristic of apoptosis-like PCD. **D**: Vacuolated IHC undergoing an autophagic process in the noise-damaged region of the organ of Corti, 6 h after acoustic trauma. Autophagic vacuoles are apparent in the cytoplasm. The mitochondria are altered (arrows) but the cytoplasmic lateral membrane is preserved without condensed chromatin. Note the swollen afferent dendrites (asterisks) at the basal pole of the IHC. **E**: A degenerating OHC (O_1) with vacuolated cytoplasm, distorted and altered mitochondria (arrowheads), an electron-dense nucleus (n) due to chromatin compaction (signs of secondary necrosis), and a disintegrated cytoplasmic membrane (arrow). **F**: A necrotic IHC (I) is characterized by cytoplasmic and nuclear swelling and cell membrane rupture. Note the swelling afferent dendrites (*asterisks) at the basal pole the sensory IHC.* ip: inner pillar cell, D: Deiter's cells, e: efferent fibers, n: nucleus. Scale bar in **A, C, D** = 6 μm**, B** = 1 μm, **E** and **F** = 10 μm.

strongly suggests that dysregulation of apoptosis is associated with aging, and apoptosis may be the most common form of cochlear cell death within the aging gerbil, mouse and rat cochlea [17, 18].

1.2. Autophagy (Type 2 PCD)

Autophagy, a lysosomal pathway for degrading organelles and long-lived proteins, is becoming recognized as a key adaptive response that can preclude death in stressed or diseased cells. It has become clear in recent years that autophagy not only serves to produce amino acids for ongoing protein synthesis and to produce substrates for energy production when cells become starved, but it is also able to eliminate defective cell structures and for this reason the process may be implicated in several diseased states. A growing amount of evidence indicates a strict causality between the reduction of autophagic functionality and aging. However, the concept of autophagy as a defense mechanism seems in contrast to evidence that it acts as a cell death pathway in some circumstances. Autophagic cell death appears to be associated with experimental and human (Alzheimer's, Parkinson's) neurodegenerative diseases [19-21]. It is important to note that autophagic cell death and apoptosis can occur in the same tissue. Morphologically, autophagy is defined by proliferation of autophagic vacuoles and progressive disappearance of organelles, but relatively well-preserved cytoskeletal and nuclear integrity until late in the process [22, 23]. Clarke proposed that neurons destined for elimination internalize cytoplasmic components into autophagic compartments to execute self-degradation. This morphological pattern, termed Type 2 PCD or autophagic cell death, suggests a mechanism distinct from Type 1 PCD or apoptosis [3].

In the inner ear, there are only three published reports on autophagy: demonstration of autophagic vacuoles in the most immature hair cells after gentamicin treatment in saccular cultures [24], in the postnatal transformation of Kolliker's organ cells in the cat [25] and in the developing inner ear of mouse embryos [26]. No evidence of autophagy has been reported in an animal model of cochlear aging.

1.3. Necrosis-Like Programmed Cell Death (Type 3 PCD)

Necrosis-like PCD (non-lysosomal vesiculate or cytoplasmic) is a caspase-independent mode of cell death with

necrotic morphology, which appears to be regulated by intrinsic cellular programs [27-30]. This form of PCD is characterized by cytoplasmic vacuolation, lack of apoptotic morphology, lack of caspase activation and lack of inhibition by caspase inhibitors (p35, zVAD.fmk, xiap, and Boc-aspartyl fmk) and Bcl-xL. In 'necrosis-like PCD' chromatin may not condense at all, but at best, chromatin clusters only to loose speckles. This active necrosis-like PCD is observed in various paradigms of cell death in conditions in which caspases are inhibited [31]. Indeed, there is increasing evidence for caspase-independent cell death [32] and caspase inhibition occasionally turns the morphology of PCD from apoptosis into necrosis without preventing death itself [33, 34]. However, these models of caspase-independent necrosis might provide potential targets for a novel cancer or neurodegenerative diseases therapy.

In the cochlea, only one study has shown the necrotic-like and apoptotic-like PCD without caspase activation in a mouse model of progressive kanamycin-induced hair cell loss [35].

1.4. Necrosis

Necrosis has been defined as non-apoptotic, accidental cell death [36]. Necrosis is considered to be a passive cellular event and may be induced by mechanical damage, lack of blood or a nutritional supply, or by exposure to certain toxic organisms, agents or chemicals. Necrosis is characterized by cellular oedema and disruption of the plasma membrane, leading to release of the cellular components and thus to an inflammatory tissue response [37, 38].

In the cochlea, necrosis has been shown after exposure to high doses of ototoxic drugs or extremely high sound intensity (at least 130 dB SPL) which causes direct mechanical destruction of the sensory hair cells and supporting cells in the organ of Corti [39, 40]. Sometimes, individual sensory hair cells share both necrotic and apoptotic features, which may be explained by the occurrence of a secondary necrotic process due to a metabolism deficiency resulting in an inability to maintain the apoptotic process in the damaged cells [29]. The presence of these different phenotypes indicates that the degeneration of sensory hair cells after ototoxic drugs or intense sound exposure implies involvement of different mechanisms of cell death [14, 41, 42]. Necrosis has only been shown in type II fibrocytes in age-related atrophy of the lateral wall in gerbil cochleae [43, 44].

2. COCHLEAR CELL DEATH PATHWAYS

The apoptotic program can be initiated by factors acting *via* an intrinsic (intracellular) or an extrinsic (extracellular) route. The intrinsic pathway of apoptosis (IPA) is one of the main apoptotic pathways. A distinctive feature of this pathway is the involvement of mitochondria. The implication of mitochondria in apoptosis has important consequences for the understanding of the normal physiology of cell death, its deregulation in cancer and degenerative diseases, and the development of novel cytotoxic and cytoprotective drugs [45, 46].

Another cell death pathway is the extrinsic signal pathway, which leads to apoptosis of cells after activation of receptors in the cell membrane that are a subset of the tumor necrosis factor receptor (TNF-R) family [47]. Both intrinsic and extrinsic pathways can be modulated by c-Jun N-terminal Kinase/stress-activated protein kinase (SAPK/JNK), calcium or reactive oxygen species (ROS) signalling pathways. Several cell death signaling pathways tested in the cochlea are shown in Fig. **2**.

2.1. Intrinsic and Extrinsic Pathways

The mitochondrial (intrinsic) pathway is activated extensively in cells in response both to extracellular signals and to internal insults like DNA damage, hypoxia, loss of survival signals, ototoxic drugs, intense sound stimulation, oxidants, Ca^{2+} overload [48-50], or during the cochlear aging process [18]. The core of this pathway is the apoptosome [51] consisting of cytochrome c (cyt.c), 20-deoxyadenosine 50-triphosphate (dATP) and the apoptotic protease activation factor-1 (Apaf-1), which promotes the assembly of a caspase-activating complex [52]. The death receptor (extrinsic) pathway, initiated by extracellular death ligands (i.e., tumor necrosis factor and Fas), activates caspases-8 and -10, which can subsequently activate downstream effector caspases (i.e., procaspase-3) [53, 54] and might also activate the mitochondrial pathway [55]. The cell death receptor and mitochondrial pathways converge at the level of pro-caspase-3 activation. Currently the role of both the mitochondrial pathway and the death receptor pathway in ototoxic-, noise-, or aging-induced cochlear cell death is slowly being elucidated (Fig. **2**).

2.1.1. Aminoglycoside-Induced Ototoxicity

Aminoglycoside antibiotics such as neomycin and gentamicin induce hair cell death that has been shown to be mediated by caspases, such as caspase-9 and caspase-3. This occurs in both the vestibular organ [9, 56] and the cochlea [8, 57]. Further evidence for involvement of the mitochondrial pathway has been obtained in a transgenic mouse that overexpresses Bcl-2, an anti-apoptotic protein. In these mice, neomycin did not activate caspase-9 in the hair cells and hair cell survival was significantly increased [50]. These data indicate that the mitochondrial pathway for apoptosis is important in aminoglycoside-induced hair cell death. These ototoxic antibiotics also activate caspase-8, a component of the death receptor pathway in the avian basilar papilla *in vitro* [57]. Cunningham *et al.* [56] show caspase-8 activation in neomycin damaged hair cells in adult utricular *in vitro*. In this utricule model, caspase-8 does not seem to be the primary caspase mediating hair cell death because inhibition of caspase-8-like activity is not sufficient to prevent either neomycin-induced hair cell apoptosis or caspase-3 activation.

2.1.2. Cisplatin (CDDP)-Induced Otoxicity

Cisplatin (CDDP) is a highly effective and widely used anti-cancer agent [58]. The risk of ototoxic and nephrotoxic side effects commonly hinders the use of higher doses that could maximize its anti-neoplastic effects [59]. Various studies have shown both an upstream initiator caspase (i.e., caspase-9) as well as a downstream effector caspase (i.e., caspase-3) were to be activated in CDDP-damaged OHCs and some IHCs located in the basal turn of the cochlea [12, 60, 61]. Devarajan *et al.* [60] reported CDDP-induced apoptosis in an immortalized cochlear cell line with an increase in

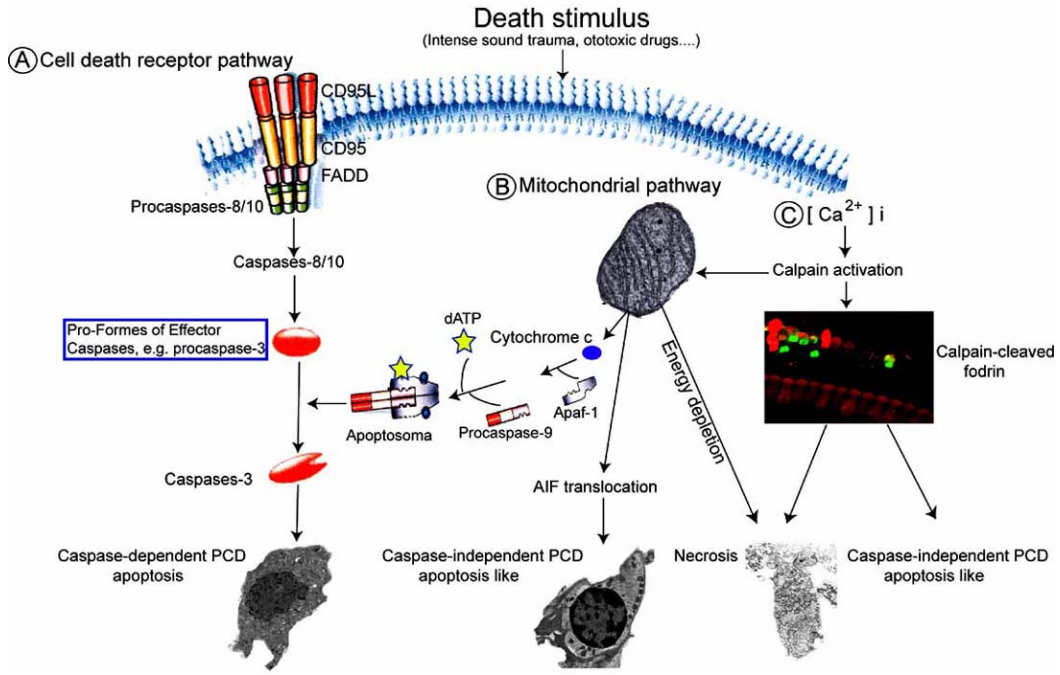

Fig. (2). A schematic presentation of the cell death signaling pathways tested in the cochlea.

A: The death receptor pathway (left side of figure) initiated by extracellular death ligands (i.e., CD95L), activates caspases-8 and -10, which can subsequently activate downstream effector caspases (i.e., procaspase-3). **B:** The mitochondrial pathway (middle side of figure) is extensively activated in cells in response to extracellular signals and internal insults (i.e., DNA damage, intense sound trauma, ototoxic drugs, or aging…). Pro-apoptotic signals induce release of cytochrome *c* and other pro-apoptotic proteins such as AIF from the mitochondria to the cytoplasm. The cytochrome *c* associates with Apaf-1 and procaspase-9 to form an apoptosome complex, which in turn activates downstream effector caspases (i.e., caspase-3). The cell death receptor and mitochondrial pathways converge at the level of pro-caspase-3 activation and contribute the caspase-dependent apoptosis. The AIF release and translocation from mitochondria to the nucleus lead to apoptosis-like PCD. Cells in which mitochondria have ruptured are at risk for death through a slower non-apoptotic mechanism resembling necrosis. **C:** Death stimuli (i.e., intense sound trauma, ototoxic drugs…) can also induce an increase of intracellular calcium in hair cells. Calcium influx can activate calpains, a family of calcium-activated proteases, which promote the breakdown of proteins (i.e., fodrin), kinases, phophatases and transcription factors and receptors and lead to necrosis and/or apoptosis-like PCD. Calpain activation might also activate the mitochondrial pathway (right side of figure). PCD: programmed cell death.

caspase-8 and caspase-9 activity suggesting that both the death receptor and the mitochondrial pathways are involved in CDDP-induced apoptosis of hair cells. In contrast Wang *et al.* [12] did not observe a significant increase in caspase-8 activation in CDDP-treated guinea pig cochleae *in vivo*. It is worth noting that immortalized auditory cell line used by Devarajan *et al*. [60] represents OHC precursors, and not adult-like OHCs. Thus, the different patterns of caspase-8 activation observed *in vitro* by Devarajan *et al*. [60] and in the *in vivo* study by Wang *et al.* [12] may be due to the differences in these two experimental models. In addition, local scala tympanic perfusion of z-IETD-fmk, a caspase-8 inhibitor, was ineffective in preventing both CDDP-induced hair cell death and hearing loss in guinea pig *in vivo* [12].

2.1.3. Intense Sound

Recent studies have shown that acoustic overstimulation induces calcium overload that triggers mitochondria-mediated death pathways through activation of Bcl-2-associated death promoter (BAD) by calcineurin [62]. Release of cytochrome c from mitochondria into the cytosol leads to the activation of caspase-9 and other downstream effector caspases such as caspase-3, and related pathways, indicating the involvement of the caspase pathway in intense sound-induced apoptosis [13, 63, 64]. The activation of effector caspases then leads to the cleavage of their substrates such as F-actin and fodrin in affected hair cells [65, 66]. Nicotera *et al.* [63] reported that noise exposure triggered activation of caspases -8 and -3 which implies that the death receptor pathway is activated in noise damaged cells. Han *et al*. [67] reported that intense noise induced the activation of caspase-independent pathways involving the translocation of endonuclease G (EndoG) and apoptosis-inducing factor (AIF) from mitochondria to the nucleus.

2.1.4. Aging

Recent studies suggest that apoptosis is involved in the aging process of various tissues. In the aged human brain, apoptotic-like changes [68] and an increased expression of Bak [69] have been reported. In peripheral blood lymphocytes from older humans, activation of both the intrinsic and extrinsic apoptotic pathways such as increased expression of Fas, Bax, and caspase-8, -9, -3 as well as decreased expression of bcl-2 [70, 71] have also been reported. In the cochlea, an increase of caspases-9, -7, -3 and Bax activity is observed within the aging rat cochlea, suggesting that age-related

apoptosis is involved in an intrinsic pathway of pro-apoptotic signalling [18].

2.2. c-Jun N-Terminal Kinase/Mitogen-Activated Protein Kinase Signalling Pathway

The c-Jun N-terminal Kinase/mitogen-activated protein kinase (JNK/MAPK), a member of the mitogen-activated protein kinase family, can be activated by a variety of environmental stresses, such as UV irradiation or exposure to toxins [72, 73]. Activated JNKs can phosphorylate a variety of cytoplasmic and nuclear proteins, i.e., c-Jun [74, 75], activating transcription factor 2 [76] and ETS-containing factors [77]. In the cochlea, JNK can be activated by various forms of insults such as loss of trophic factor support [78], drug ototoxicity and intense sound exposure [14, 79].

2.2.1. Ototoxic Drugs

The ototoxic aminoglycosides have been shown to activate the JNK signalling pathway and induce cochlear cell death both *in vitro* and *in vivo* [14, 79]. In contrast, it appears to play a protective role against DNA damage-induced apoptosis, as shown by recent studies which have demonstrated that the JNK signal cascade is activated by CDDP and is necessary for the repair of DNA-CDDP adducts and for cell viability following CDDP-treatment [12, 80, 81].

2.2.2. Intense Sound

JNK can also be activated by intense sound exposure [14, 49, 79]. Sound trauma induced a significant increase in phosphorylation of the nuclear transcription factor c-Jun, activation of Bax, release of cytochrome c and cleavage of fodrin by activated effector caspases [49]. Taken together, these results suggest that JNK activates the intrinsic mitochondrial cell death pathway, and that this pathway is one of the major intracellular cascades by which hair cells are damaged in response to intense sound-induced damage.

2.2.3. Aging

It has been reported that the basal level of activity of the stress-activated pathways increases, thereby becoming a basic factor in the development of a state of chronic stress in aged tissues [82-85]. There have been found to be age-associated changes in the basal levels of phosphorylation and kinase activities of the p38 MAPK and SAPK/JNK stress signalling proteins in aged rodent liver and brain tissues [84, 85]. Furthermore, the upstream activators of p38 MAPK, i.e., MKK3, and its downstream target, ATF-2, are all increased in the aged mouse liver [85]. These studies have established that aging affects the basal level of stress response signalling pathways. However, the role of these stress response pathways during the cochlear aging process has not been shown to date.

2.3. Calcium, Calpains and Cathepsins

It has long been known that an increase in the intracellular concentration of calcium is lethal to cells. For example, in the cochlea, the drug thapsigargin, which blocks the transport of calcium into intracellular storage sites and increases levels of free intracellular calcium results in hair cell loss [86]. The mechanism for this hair cell death appears to be the activation of at least three proteases including caspases, calpains and cathepsins, which are all induced by the increased intracellular levels of calcium [87-89]. Calpains are calcium-dependent cysteine proteases that are involved in cellular function and have been implicated in various diseases [87, 90]. For example, calpains will cleave cytoskeletal proteins, signal transduction proteins, apoptotic regulatory factors and caspases leading to cell death by apoptosis or necrosis [87, 90]. In addition, calpain activation has been implicated in excitotoxic events triggered by nitric oxide [91]. Activation of calpains will also damage lysosomal membranes resulting in the release of lysosomal proteolytic enzymes called cathepsins [90]. Cathepsins will induce non-specific cleavage of various intracellular proteins and they have been implicated in both apoptosis and necrosis [87].

2.3.1. Ototoxic Drugs

Several studies suggest that calpains and cathepsins may have a role in ototoxic drug- induced cell death in the cochlea. An increase in cytosolic Ca^{2+} level is required for calpain activation [92]. Ca^{2+} level increase has been found in damaged hair cells of the mammalian vestibular end-organs and in mature avian auditory epithelium after aminoglycoside treatment *in vivo* [93, 94], Ladrech *et al.*, [95] showed an abnormal accumulation of fodrin breakdown products, specifically produced by calpain activity, in amikacin damaged OHCs and IHCs of adult rats that have been treated during neonatal period. Mandic *et al.*, [96] reported on the possible involvement of calpains in CDDP toxicity in a human melanoma cell line suggesting a role for calpains in CDDP ototoxicity. However, the lack of a protective effect of calpain inhibitors on CDDP toxicity in cultured auditory hair cells and neurons argues against this hypothesis [97].

2.3.2. Intense Sound

Intracellular calcium and calcium buffering mechanisms are implicated in intense sound-induced cochlear cell damage. Studies have shown that sound overstimulation increases intracellular calcium in sensory hair cells *in vitro* [98] and causes an increase in calpain immunoreactivity *in vivo* [99]. Furthermore, the accumulation of calpain-cleaved fodrin was observed in hair cells damaged by sound trauma, suggesting an involvement of calpains in the degeneration of hair cells induced by sound trauma [49]. It is well known that intense sound-induced excessive glutamate release in the cochlea can induce excitotoxicity of auditory neurons by over stimulation of glutamate receptors, which in turn leads to calcium overload in the postsynaptic neuron [100]. Indeed, intracochlear perfusion of glutamate antagonists during intense sound exposure prevents 50% of the acute threshold elevation by protecting the auditory neuron nerve endings [101].

2.3.3. Aging

One of the common features of cells from senescent tissues is the accumulation of abnormal proteins. Several hypotheses have been proposed to explain this phenomenon. A defect in proteolytic systems such as the calcium-activated calpain pathways and multiple lysosomal pathways usually responsible for the elimination of altered proteins from the cells could clearly contribute to such accumulation. The activity of these pathways significantly decreases with age, and

this decrease might account for the cytosolic accumulation of aberrant substrate proteins in senescent cells. Unfortunately, the roles of these pathways in cochlear aging processes still need to be elucidated.

2.4. ROS Pathways

Mitochondria serve as the major energy-producing powerhouse, whereby the generation of ATP is associated with the utilization of molecular oxygen. A significant fraction (2–3%) of molecular oxygen consumed by mitochondria may be reduced in a one-electron fashion to yield a series of reactive oxygen species (ROS). ROS are capable of damaging components of the electron transport apparatus and can, in turn, disrupt mitochondrial functioning, limiting cellular ATP levels and ultimately resulting in cell death.

2.4.1. Ototoxic Drugs

Generation of ROS and other free radicals is one of the mechanisms by which aminoglycosides or cisplatin cause apoptosis of sensory hair cells [102-106]. Several reports have placed mitochondrial homeostasis at the core of aminoglycoside-induced ototoxicity [107, 108]. Although it has only been postulated that free radical formation causes ototoxicity, this hypothesis has strong support from the results of experiments which demonstrate that treatment with antioxidants attenuates aminoglycoside-, or cisplatin-induced hearing loss [104, 105, 109].

2.4.2. Intense Sound

Experimental observations support the importance of reactive oxygen species in intense sound-induced hearing loss [110-113]. Superoxide anion radicals appear in the stria vascularis after intense sound exposure [114], hydroxyl radicals significantly increase in the cochlea with intense sound [115], glutathione increases in the lateral wall following intense sound exposure [116], glutathione peroxidase and malondialdehyde activity increase progressively with the severity of intense sound [117, 118]; isoprostanes, directly reflecting ROS formation after intense sound, form in the organ of Corti and lateral wall [119]; and oxidative-induced DNA damage follows intense sound exposure [120]. These reactive oxygen species (ROS) may directly destroy DNA and cell membranes, as well as acting as signalling molecules for the upregulation of apoptotic cell death genes. ROS can directly cause cell damage by reacting with proteins, lipids, and DNA. ROS reactions with the plasma membrane lead to formation of phospholipid membrane peroxidation products such as 4-hydroxy-2-noneal (4-HNE), which is highly reactive and can lead to neural apoptosis [121]. 4-HNE formation has been demonstrated in both auditory neurons and hair cells following intense sound exposure [122]. Recent studies have suggested that 4-HNE-induced JNK activation promotes its translocation to the nucleus, where JNK-dependent phosphorylation of c-Jun and the transcription factor activator protein (AP-1) binding takes place [123, 124]. Importantly, JNK activation by 4-HNE also leads to the activation of caspase-3 [124].

2.4.3. Aging

The free radical theory of aging proposes that endogenously produced oxygen radicals are a basic cause of the progressive age-associated decline in tissue function, and that oxidative stress generated by extrinsic environmental factors accelerates this decline [125-128]. More precisely, the theory postulates that age-associated accumulation of oxidative damage to DNA, RNA, proteins and lipids leads to the development of biochemical characteristics of aged tissues [129-132]. In fact, the accumulation of ROS damaged macromolecules (i.e., nuclear and mitochondrial DNA (mtDNA), and carbonylated proteins) is an indication of the oxidative damage that can affect mitochondrial function [130, 133-135]. Consequently damaged mitochondria release more ROS, initiating a self perpetuating cycle of increasing damage to macromolecules [133-135]. These insults render the aged cell more vulnerable to extrinsic environmental factors and disease, and are therefore important causative factors in the gradual decline in tissue function [125, 126].

It has been showed that reactive oxygen species (ROS) are implicated in hearing loss associated with aging [136-139]. Superoxide dismutases (SODs) form a first line of defense against damage mediated by the superoxide anion, the most common ROS. Absence of Cu/Zn SOD (SOD1) has been shown to potentiate hearing loss related to age. Oxidative stress has also been involved in presbycusis by reports of increased susceptibility of SOD1 knockout mice [140, 141] and increases of SOD1 mRNA in older mice [139]. A recent study in the aging mouse cochlea described an increase in various forms of oxidative stress such as lipid peroxidation and glutathionylation, whereas cellular antioxidant defense systems such as AIF and SOD2 are reduced [142]. Furthermore, a diet containing antioxidants reduced the magnitude of cochlear degeneration in aged dogs [143].

This overview of the literature shows the complexity of the hair cell death pathways involved in ototoxic drug-, intense sound-exposure and aging-induced hearing loss. Note however that the data reported herein come from different *in vitro* (immortalized cell lines, acute and cultured isolated hair cells and auditory neurons, cochlear and utricule explant from neonates or adults animals etc.) and *in vivo* models (adult guinea pigs, rat, gerbil, transgenic mice, chicken, etc.) and different stress situations (ototoxic drugs, sound trauma, aging). This is an important point in terms of the potential applicability to clinical situations since a drug can have a protective effect only in one stress situation or one particular model. One example is the case of the peptide inhibitor D-JNKI-1, which protected the cochlea against sound trauma, but aggravated the ototoxic effect of CDDP [12, 14]. Thus, it is not reasonable to extrapolate results obtained in one particular pathological condition to others. In the same line of arguments, the choice of the adequate animal model is crucial to the transfer of pre-clinical trials to the human clinic.

3. PHARMACOLOGICAL STRATEGIES

Understanding the molecular mechanisms of sensory hair cell death allows us to focus on therapeutic strategies to protect the cochlea against drug and intense sound exposure or age-induced hearing loss. In this section we discuss strategies based on the inhibition of regulators or executors of apoptotic sensory hair cell death pathways or the use of antioxidants and free radical scavengers.

3.1. Inhibition of JNK/MAPK Signalling Pathway

3.1.1. Ototoxic Drugs

CEP-1347 is a derivative of indolocarbazole K252a that blocks the MAPK signal pathway at the level of mixed lineage kinases (MLK3). A study using CEP-1347 [79] show that blocking the MAPK/JNK cell death signal cascade is a highly otoprotective treatment in preventing neomycin-induced loss of sensory hair cells from neonatal rodent organ of Corti explants. Systemic treatment with CEP-1347 can partially protect the auditory sensory hair cells from the ototoxic effects of gentamicin [144], and can thus preserve hearing thresholds.

JNK-interacted protein-1 (JIP-1) is a scaffold protein located in a cell's cytoplasmic matrix that retains members of the MAPK-JNK signal cascade [145]. Wang *et al.* [14] used a cell penetrating JNK inhibitor, D-JNKI-1 peptide, which was constructed by linking the 20 amino acid segment of a JNK-binding motif from JIP-1 to a 10 amino acid HIV-TAT transporter sequence and by synthesizing a highly protease resistant form (retro-inverso construction) of the JNK binding domain that doubles its intracellular half-life [145]. This study demonstrated that inclusion of D-JNKI-1 in the culture medium protected sensory hair cells in P-3 mouse organ of Corti explants against the ototoxic effect of neomycin. Additionally, the action of D-JNKI-1 in these explant cultures also prevented phosphorylation of c-Jun within sensory hair cell nuclei and inhibited the upregulation of c-fos mRNA production that occurs within neomycin-exposed cochlear explants. The results of *in vivo* experiments reported in this paper demonstrate that perfusion of the guinea pig's scala tympani with D-JNKI-1 effectively prevented both loss of sensory hair cells and the development of a permanent hearing loss induced by neomycin ototoxicity (Fig. **3**). Interestingly, JNK inhibitors can have the opposite effect depending on the individual ototoxic drug. For example, while D-JNKI-1 did protect the cochlea against neomycin (Fig. **3**), it exacerbated the otoxicity of cisplatin (Fig. **4**) [12]. This suggests that the JNK pathway is not involved in cisplatin-induced hair cell death, but instead may have a role in DNA repair and maintenance of cisplatin-damaged sensory cells.

3.1.2. Intense Sound

Systemic administration of CEP-1347 provided partial protection against sound trauma- induced hearing loss [79]. When applied directly into the cochlea, D-JNKI-1 almost completely protected the cochlea against permanent hearing loss induced by sound trauma. Similar results were obtained when D-JNKI-1 was applied onto the guinea pig round window membrane *via* an osmotic minipump [49]. FITC-conjugated D-JNKI-1 peptide applied onto an intact cochlear round window membrane diffused through this membrane and penetrated all cochlear tissues with the exception of the stria vascularis. A time-sequence of fluorescence measurements demonstrated that FITC-labelled D-JNKI-1 remained in cochlear tissues for as long as 3 weeks. In addition to blocking JNK-mediated activation of a mitochondrial cell death pathway, round window membrane-administered D-JNKI-1 prevented hair cell death and development of a permanent shift in hearing threshold caused by sound trauma in a dose-dependent manner (Fig. **5**). The therapeutic window for protection of the cochlea from sound trauma with round window membrane delivery of D-JNKI-1 extended out to 12 hours post- sound exposure (Fig. **5**). Whereas the role of the JNK/MAPK pathways in ototoxicity and sound trauma is well documented, its implication during cochlear aging processes needs to be elucidated in the future.

3.2. Caspase inhibitors

3.2.1. Ototoxic Drugs

Blocking events later in the apoptotic pathway also provides protection from ototoxic drugs. When mouse utricles were treated with neomycin and an inhibitor of caspase-9 *in vitro*, sensory hair cell death was reduced [56]. Treatment with a broad-spectrum pancaspase inhibitor (i.e., either z-VAD-fmk or BAF) has been shown to provide a significant level of protection against aminoglycoside-induced hair cell death in the vestibular organ [146]. Caspase inhibitors offered significant protection from cisplatin-induced apoptosis for both the hair cells in organ of Corti explants and the neurons in the dissociated spiral ganglion cell cultures [8]. Moreover, intracochlear perfusion of a caspase-3 inhibitor (z-DEVD-fmk) and a caspase-9 inhibitor (z-LEHD-fmk) dramatically reduced the ototoxic effects of CDDP, as evidenced by a lack of DNA fragmentation with almost no apoptotic cell death of hair cells or other cell types within the cochlea and almost no loss of hearing [12]. Finally, *in vivo* injection into the round window of AAV harbouring a gene encoding a caspase inhibitor XIAP (X-linked inhibitor of apoptosis protein) protected against cisplatin ototoxicity in the rat [147].

3.2.2. Intense Sound

The apoptotic hair cell death in the noise-damaged cochlea was found to be associated with activation of caspase-3 [65], consequently intracochlear perfusion of 200 μM of the caspase-3 inhibitor Z-DEVD-FMK prevented noise-induced F-actin cleavage in the outer hair cells of chinchilla cochleae [65].

3.2.3. Aging

It is well known that apoptosis increases with aging. However it remains to be elucidated whether specific inhibitors of apoptosis can play a role in preventing the loss of cells in aging tissues. Several mammalian inhibitors of apoptosis proteins (IAPs) have been discovered, one example being X-linked inhibitor-of-apoptosis protein (XIAP). XIAP can bind to caspase-3 and inhibit its protease activity. Recently, it has been shown that over-expression of XIAP by a transgenic mouse delayed the age-related hearing loss [148].

3.3. Calpain Inhibitors

Early work on calpain inhibitors was limited to protein inhibitors and other nonselective enzyme inhibitors. Peptidyl aldehydes such as leupeptin and antipain are also among the earliest reported calpain inactivators. A variety of calpain inhibitors are under development. From a therapeutic perspective, calpain inhibitors may have several advantages, since calpain proteolysis represents a later component of a

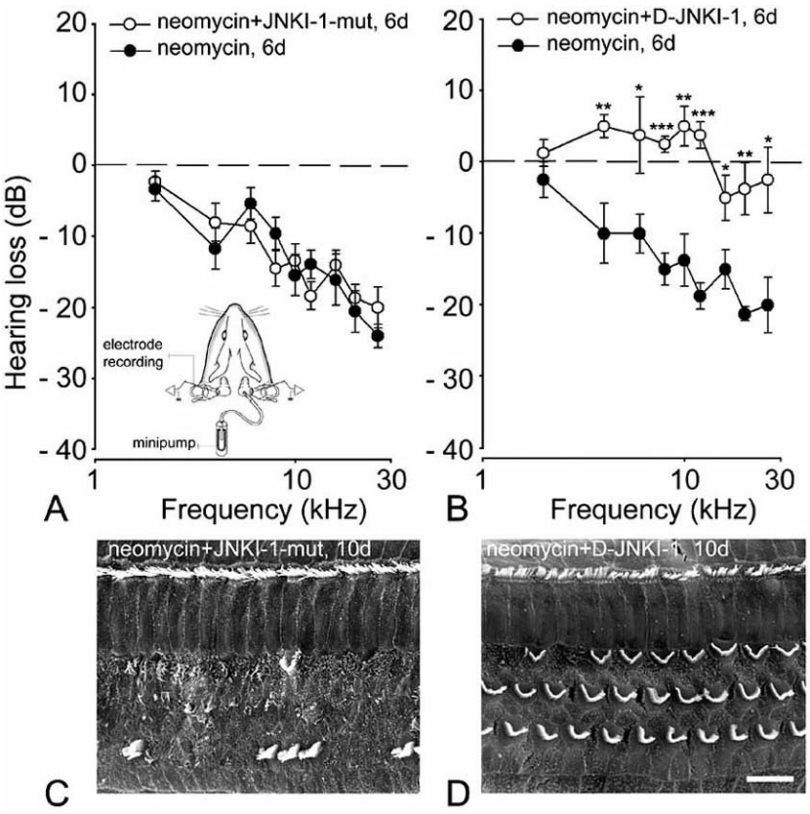

Fig. (3). Intracochlear perfusion with D-JNKI-1, but not with inactive JNKI-1-mut peptides, protects against neomycin–induced hearing loss. A comparison of local delivery of JNKI-1-mut and D-JNKI-1 on neomycin-induced hearing loss. Hearing loss was calculated as the difference in dB between auditory thresholds before treatment, and after the 5-day period of neomycin treatment. Results are shown as the mean ± SEM. All animals were treated with 5 sequential doses (300mg/kg i.p. per day) of systemic neomycin. A: A comparison of 10 μM JNKI-1-mut-perfused right cochleae (white circles) with contralateral non-perfused left cochleae (black circles) in the same neomycin-treated animals (n=6). No intra-aural differences were noted, i.e., both ears showed the same level and pattern of hearing loss. B: A comparison of 10 μM D-JNKI-1-perfused right cochleae (white circles) with non-perfused left cochleae (black circles) in the same neomycin-treated animals (n=6). N.B. no hearing loss occurred in the D-JNKI-1-perfused cochleae. C and D: Scanning electron micrographs from the basal turns of a cochlea of a neomycin-exposed animal. Note the extensive loss of OHCs from the organ of Corti of the basal turn of the 10 μM JNKI-1-mut-perfused, neomycin-exposed right cochlea (in C). Only six OHCs remain in the area viewed in C as apposed to the 29 OHCs present in the image presented in D, in which perfusion of a 10 μM solution of D-JNKI-1 prevented loss of OHCs. O, Area of all three rows of OHCs. I, Single row of IHCs. Scale bar, C and D = 15μm. Inset: Experimental protocol. General view of the monitoring system and surgical approach. Each cochlea was exposed via a postauricular approach. The right cochlea was implanted with a minipump and a recording electrode was placed on the round window membrane of both left and right cochleae (Adapted from Eshraghi *et al*., and Wang *et al*.) [14, 190].

pathway mediating cell death initiated by excitotoxicity and elevated Ca^{2+} levels.

3.3.1. Ototoxic Drugs

Leupeptin, an inhibitor of calpains and cathepsins, has been shown to inhibit programmed cell death in gentamicin and neomycin damaged sensory hair cells [149, 150]. The addition of 1 mM of leupeptin significantly reduced sensory hair cell loss in the crista and utricle cultures that had been exposed to gentamicin concentrations ranging from 0.1 to 3 mM [149]. These results suggest that one of the early steps in gentamicin ototoxicity may involve calcium-activated proteases that lead to the demise of cochlear and vestibular hair cells. However, the calpain inhibitors are not effective against CDDP or carboplatin ototoxicity on sensory hair cell and auditory neurons *in vitro* or on sensory hair cells *in vivo* [99, 97].

3.3.2. Intense Sound

Calcium buffering mechanisms are implicated in cochlear cell damage that has been induced by sound trauma. Consistent with this, when the calpain inhibitor leupeptin was infused into the basal turn of the chinchilla cochlea, it reduced sensory hair cell loss following a 105 dB SPL exposure by as much as 60% [99]. In addition, infusion of BN82270, a novel dual inhibitor of calpains and of lipid peroxidation [151] into the scala tympani of the guinea pig cochlea prevented the formation of calpain-cleaved fodrin, DNA fragmentation and hair cell degeneration caused by sound trauma. This was confirmed by functional tests *in vivo*, showing a clear dose-dependent reduction of permanent hearing loss.

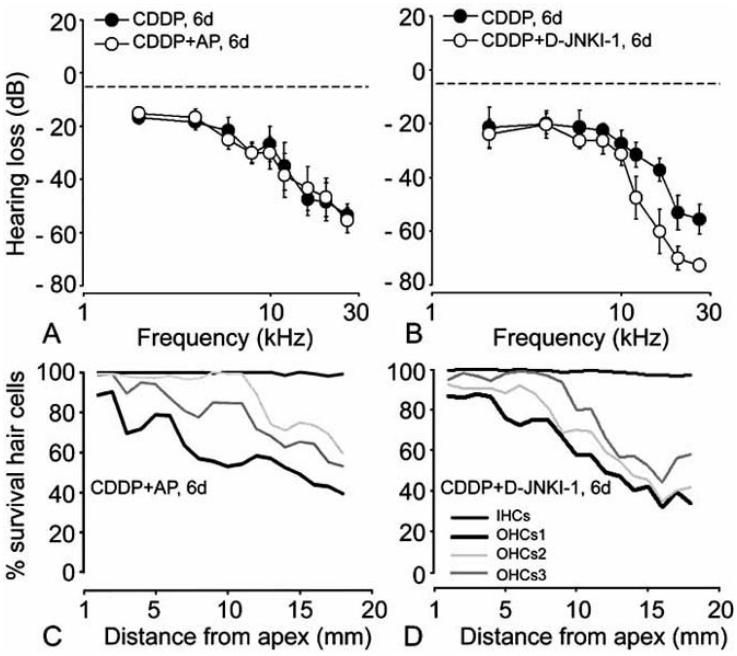

Fig. (4). A comparison of local delivery of artificial perilymph and D-JNKI-1 on CDDP-induced hearing loss. Hearing loss was calculated as the difference in dB between auditory thresholds before treatment, and after the 5-day period of CDDP-treatment. Results are shown as the mean ± SEM. All animals were treated with 5 sequential doses (2mg/kg i.p. per day) of systemic CDDP. **A**: A comparison of artificial perilymph (AP)-perfused right cochleae (white circles) with contralateral non-perfused left cochleae (black circles) in the same CDDP-treated animals (n=6). No differences were noted, i.e., both ears show the same level and pattern of hearing loss. **B**: A comparison of D-JNKI-1-perfused right cochleae (white circles) with non-perfused left cochleae (black circles) in the same CDDP-treated animals (n=6). Hearing loss was similar between 2 and 10 kHz, but was greater in the D-JNKI-1-perfused cochleae between 10 and 26 kHz ($p<0.05$). **C** and **D**: Cytocochleograms showing mean hair cell loss in artificial perilymph (AP)-perfused cochleae ($n = 3$) (**C**) and D-JNKI-1-perfused cochleae ($n = 3$) (**D**). These graphs demonstrate the percentage of surviving IHCs and OHCs as a function of the distance from the apex (mm). Note the extensive loss of OHCs and a few IHCs in the basal and middle turns of the both artificial perilymph - and D-JNKI-1-perfused cochleae (Adapted from Wang *et al.*)[12].

Furthermore, BN82270 still remained effective even when applied onto the round window membrane after sound trauma had occurred, within a therapeutic window of 24 hours. This indicates that BN82270 may be of potential therapeutic value in treating the cochlea after sound trauma [66].

3.3.3. Aging

Efficiency of calcium storage alters with age, and this may explain the increase in resting intracellular Ca^{2+} levels in senescent animals [152, 153]. Increased intracellular Ca^{2+} concentrations can significantly contribute to increased susceptibility to apoptosis *via* activation of calpains [154, 155]. The protective effect of calpain inhibitors to slow down hearing loss associated with age needs to be further evaluated.

3.4. Antioxidants, Free-Radical Scavengers

3.4.1. Ototoxic Drugs

A protective effect of iron chelators against gentamicin ototoxicity has been successfully demonstrated in the guinea pig *in vivo* [156]. The same authors also showed that dihydroxybenzoate protected against hearing loss mediated by other aminoglycosides as well as streptomycin-induced vestibulotoxicity. Deferoxamine, another iron chelator, also protected against cisplatin-induced oto- and nephrotoxicity [157]. *In vivo* gluthatione levels in the inner ear correlate with sensitivity to gentamicin and cisplatin ototoxicity [158]. Several studies have shown that D-methionine, 4-methylthiobenzoic acid, N-acetylcysteine, and diethyldithiocarbamate, provide complete protection from cisplatin ototoxicity [105, 106, 159, 160]. Furthermore, the administration of other antioxidant agents, including glutathione ester, reduced cisplatin ototoxicity [161]. In addition 4-methylthiobenzoic acid and D-methionine can protect against cisplatin-induced changes in cochlear function and in the antioxidant system of rats or chinchillas [106, 161, 162].

Finally, we have previously shown [163] that sodium thiosulfate (STS) protected the cochlea against CDDP toxicity in guinea pig. An explanation for this protective effect would be that the sulphur-containing (thio) compound mimics glutathione and therefore scavenges intracellular reactive oxygen species [164].

3.4.2. Intense Sound

Exogenous free radical scavengers (i.e., glutathione, deferoxamine, mannitol, allopurinol diethyldithiocarbamate, 4-methylthiobenzoic acid and ebselen) and iron chelators (i.e., deferoxamine) can limit intense sound or exposure induced-hearing loss [113, 165-169]. Kopke *et al.* [170] have demonstrated that a combination of antioxidant compounds (salicylate and N-L-acetylcysteine) reduced permanent threshold

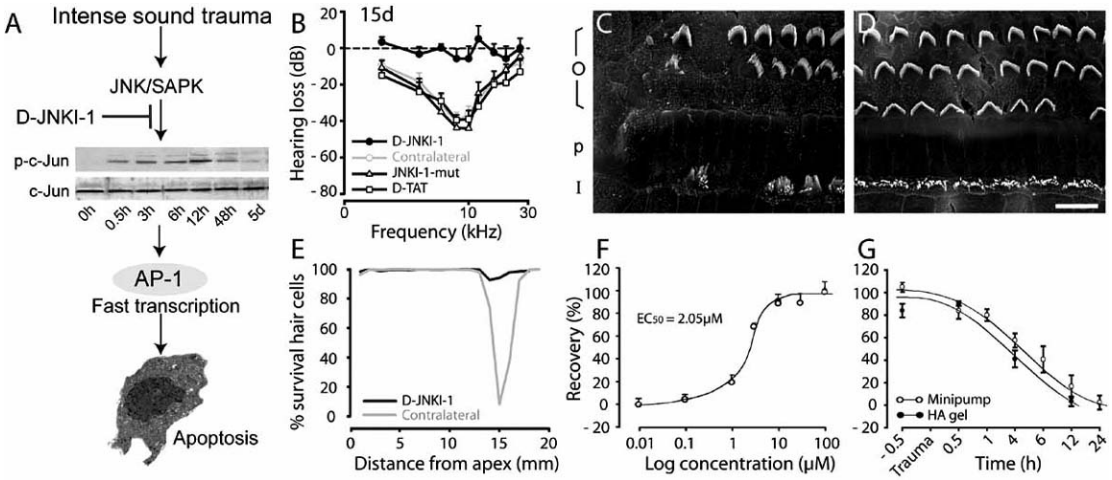

Fig. (5). D-JNKI-1 protects the cochlea against sound-induced hair cell loss and elevation of auditory threshold. A: A schematic presentation of the JNK pathways tested in the cochlea. JNK can be activated by intense sound exposure, which then induced a significant increase in phosphorylation of the nuclear transcription factor c-Jun and fast transcription of AP-1 (activator protein-1). The activation of JNK pathway can be block by using D-JNKI-1, a chemically synthesized cell permeable JNK-ligand. **B**: Hearing thresholds measured at 15 days post exposure from contralateral untreated, sound-exposed, left cochleae (grey circles) and the sound-exposed right cochleae that were infused with D-JNKI-1 (black circles), JNKI-1-mut (black triangles) or D-TAT (black squares) onto the round window membrane (RWM). Contralateral untreated cochleae had a permanent hearing loss of 40 dB SPL (grey circles). Hearing loss was not significantly different in sound-exposed cochleae treated with D-TAT or JNKI-1-mut. In contrast, a significant improvement in the recovery of hearing threshold from an initial temporary threshold shift was seen in the D-JNKI-1 treated cochleae (black circles). **C** and **D**: Scanning electron micrographs of the area of greatest damage in untreated and D-JNKI-1 treated cochleae from the same animal. Untreated cochlea showed severe damage to the IHCs (I) and to the first row of OHCs (O) with a gradation of damage extending to the second and the third rows of OHCs (in **C**). Application of D-JNKI-1 onto the RWM effectively prevented nearly all sound trauma-induced hair cell loss (in **D**). **E**: Cochleograms representing the mean survival of hair cells as a function of the distance from the apex (in mm) in contralateral untreated cochleae (grey line) and in D-JNKI-1 treated cochleae (black line). In the sound-exposed untreated cochleae, more than 80% of the hair cells were missing in the maximumally damaged area of the cochlea (14-16 mm from the apex). In contrast, less than 15% of the hair cells were lost in D-JNKI-1 treated cochleae. **F**: Dose-response curve of D-JNKI-1 efficacy at day 15 post-exposure. Hearing thresholds in response to an 8 kHz pure tone stimulus were expressed as the percentage of recovery. Dose-response data were fitted to a curve using a non-linear least-square logistic equation and the Boltzman equation was used for fitting the sigmoid curves. The EC_{50} was calculated as 2.05 μM for RWM delivery of D-JNKI-1. **G**: Time-dependent effect of RWM administration of D-JNKI-1 *via* an osmotic minipump (open circles), or *via* hyaluronic acid gel (HA gel; solid circles) on functional recovery of hearing thresholds. D-JNKI-1 treatments were commenced 0.5 before or 0.5, 1, 4, 6, 12 or 24 hrs after sound exposure. Note the efficacy of D-JNKI-1 when treatment was started within 6 hrs after the initial trauma. There is no significant difference in effectiveness with method of delivery, i.e., osmotic minipump/RWM *vs*. HA gel/RWM. Scale bars: **C** and **D** = 10 μm (Adapted from Wang *et al.*, 2007) [49].

shifts by 75% at 1 to 2 kHz, but offered no protection against hair cell loss when applied 1 hour after the sound exposure. Yamashita *et al.* [171] showed a much longer period of efficacy (a 3 day therapeutic window) when using a combination of antioxidants (salicylate and trolox) against sound trauma.

3.4.3. Aging

Reactive oxygen species (ROS) are responsible for progressive insults on mitochondria and other cellular structures, and with time these insults accumulate leading to cellular demise and resultant senescence. Recent work studying the effects of mitochondrial metabolites on aging has shown that acetyl L-carnitine and alpha-lipoic acid delay the progression of age-related hearing loss by protecting cochlear mitochondrial DNA from oxidative damage [137].

4. CLINICAL APPLICATIONS

4.1. Anti-Apoptotic Drugs

The ability to understand and manipulate the cell death machinery is an obvious goal of medical research. Novel therapeutic approaches to modulate disease by regulating apoptosis are being tested in preclinical and clinical settings. Of the variety of compounds discovered in this category, few have been discovered in preclinical or clinical settings. The lack of specific potent non-peptide apoptosis inhibitors has limited for a long time the clinical investigation of this target. In the last few years, the renewed interest of pharmaceutical companies has led to strong motivation for further research in this area [172, 173].

4.1.1. Caspase Inhibitors

The caspase family of cysteine proteases has been implicated in a wide variety of disease processes. Now more than ten caspase inhibitors are presently at various phases of clinical development/trials for various diseases [174, 175]. For example, the potent broad spectrum caspase inhibitor IDN-6556 has recently received the Orphan Drug label from the U.S. Food and Drug Administration (FDA) for use in the treatment of the patients undergoing liver transplantation and other solid organ transplantation. IDN-6556, which blocks

apoptosis, is now in a Phase II clinical trial in patients undergoing liver transplantation [175, 176], and in a phase I trial for the treatment of liver disease. Results obtained from the latter study showed that oral IDN-6556 administration significantly lowered aminotransferase activity in patients with chronic hepatitis C and appeared to be well tolerated [177].

4.1.2. Calpain Inhibitors

The calpains represent a well-conserved family of calcium-dependent cysteine proteases. Inappropriate activation of calpains is associated with several important human pathological disorders. Several classes of inhibitors, including peptidyl epoxide, aldehyde, and ketoamide inhibitors, targeting the active site of calpains have proven their efficacy in animal models of human diseases [178, 179]. However, a major limitation for the clinical use of such inhibitors is their lack of specificity among cysteine proteases and other proteolytic enzymes [180]. Recently, calpain inhibitors have being examined in a clinical trial for treating traumatic brain injury patients [181]. Up to now, no data is available on their efficacy in clinical trials.

4.1.3. JNK/MAPK Inhibitors

The robust neuroprotective activity of CEP-1347, an inhibitor of the MLK family of JNK pathway activators has been demonstrated in many *in vitro* and *in vivo* models of neuronal cell death [182, 183]. CEP-1347 is now in phase II/III clinical trials for Alzheimer's disease and Parkinson's disease [182]. Unfortunately, data from large and well-controlled clinical studies in early Parkinson's disease, showed CEP-1347 to be ineffective [183, 184]. The fact that CEP-1347 failed to be effective in human disease, in this case, Parkinson's, suggests that the animal models used may have major limitations. There is an urgent need to systematically develop models that closely mimic the time course and the physiopathology of human diseases.

Direct inhibitors of JNK activity, i.e., D-JNKI-1, have been shown to prevent hearing loss induced by intense sound and neomycin exposure in animal models [14, 49]. This compound is now in phase I/II clinical trials for acute acoustic trauma in Germany [185]. Preliminary results in 11 patients indicate that intratympanic treatment with D-JNKI-1 in patients with acute acoustic trauma could have beneficial effects against sound trauma [185].

4.2. Antioxidants, Free-Radical Scavengers

Clinical investigations with antioxidant therapy has been pursued for quite some time. N-acetylcysteine (NAC), an antioxidant and a substrate for glutathione synthesis, has been used in clinical settings as an antidote to acetaminophen overdose and mucolytic agent with FDA approval for several decades [186]. To date several preliminary clinical trials have either been completed or initiated to look at the safety and efficacy of NAC in reducing noise-induced hearing loss [187], and preventing gentamicin-induced ototoxicity in hemodialysis patients [188]. The initial clinical reports are encouraging in terms of safety and positive biological activity in reducing noise- or gentamicin-induced hearing loss [187, 188]. In addition, the antioxidant properties of salicylate, seems to improve gentamicin-induced hearing loss based on one human clinical trial [189].

CONCLUSION

This review highlights recent advances in understanding the molecular mechanisms of cochlear cell degeneration and in the directions of ongoing research aimed at the development of new pharmacologic therapies. Here, we reviewed ototoxic drugs, intense sound or aging processes activating standard apoptotic pathways, but also several additional enzymatic pathways such ROS or calcium pathways. Many of the pathways interact with each other, which is a challenge for those researchers attempting to elucidate pathways involved in response to any given stress stimulus. The fact that exogenous application of pharmacological compounds can prevent sensory hair cell death and restore auditory function in animal models provides an exciting and novel direction for investigations aimed at slowing down or stopping degenerative process of the inner ear sensory epithelium.

Of course, a lot of fundamental research still needs to be done in order to discover more potent and specific therapeutic agents. However, the first clinical trials with pharmacological compounds such as NAC or D-JNKI-1 attest that treatment of the inner ear can become a clinical reality, even if the tolerability, toxicity and efficacy of these compounds still needs to be clearly determined. In addition to proving these concepts, these compounds will need to be tested in large populations of patients with well characterized cochlear pathologies. Finally, the last and perhaps most important limitation for inner ear treatment concerns the understanding of both the nature and origin of cochlear pathologies, including genetic, sudden or age-related deafness.

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