Oxidative Stress in the Cochlea: An Update

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Abstract: This paper will focus on understanding the role and action of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in the molecular and biochemical pathways responsible for the regulation of the survival of hair cells and spiral ganglion neurons in the auditory portion of the inner ear. The pivotal role of ROS/RNS in ototoxicity makes them potentially valuable candidates for effective otoprotective strategies. In this review, we describe the major characteristics of ROS/RNS and the different oxidative processes observed during ototoxic cascades. At each step, we discuss their potential as therapeutic targets because an increasing number of compounds that modulate ROS/RNS processing or targets are being identified.

Keywords: Reactive oxygen species, ototoxicity, aminoglycoside, cisplatin, noise, presbycusis, cochlea.

Hearing functions require intricate interactions between specialised cells of the inner ear. The organ of Corti is a sensory epithelium composed of a highly ordered array of sensory hair cells and non-sensory supporting cells. Hair cells are directly connected to primary auditory neurons in the spiral ganglion. In mammals, mature hair cells and spiral ganglion neurons do not have the ability to regenerate, and their loss results in permanent hearing deficits,

1. ROLE OF ROS/RNS IN PHYSIOLOGICAL AND PATHOLOGICAL CONDITIONS

Both aerobic and anaerobic metabolism are accompanied by the production of reactive oxygen (ROS) and/or reactive nitrogen species (RNS), and organisms ranging from prokaryotes to mammals have evolved an elaborate and redundant complement of defences to confer protection against oxidative and nitrosative insults. Compelling data also indicate that ROS and RNS, at relatively low concentrations, are employed in physiological settings as signalling molecules in control of cell and tissue homeostasis, cell division, migration, contraction, and mediator production [1-6]. These signalling oxidants, which include nitric oxide (NO), superoxide radical ($O_2^{\cdot-}$), peroxynitrite ($ONOO^-$), S-nitrosothiols (RSNOs) and hydrogen peroxide (H_2O_2), are produced mainly by NADPH-dependent enzymes whose expression is tightly regulated. These enzymes function with the coenzyme nicotinamide adenine dinucleotide phosphate (NADPH), an electron scavenger. ROS are species of oxygen, which are in a more reactive state than molecular oxygen, and in which the oxygen is reduced at varying degrees. During the normal course of metabolism, electrons carried by the electron transport chain in the mitochondrion, can leak out of the pathway and interact with O_2, producing$O_2^{\cdot-}$. Other sources of $O_2^{\cdot-}$ include endogenous enzymes, such as plasma NADPH oxidases or NOXs, cytoplasmic xanthine oxidase or cytochrome P-450 isozyme in the endoplasmic reticulum. NOX3 is specifically expressed in the sensory epithelium of the inner ear and in spiral ganglion neurons. NOX3 produces physiological amounts of $O_2^{\cdot-}$ essential for its development [7,8]. Further reduction of oxygen, from the dismutation of $O_2^{\cdot-}$, produces H_2O_2 spontaneously (especially at low pH) or catalysed by superoxide dismutase (SOD). Under physiological conditions, once $O_2^{\cdot-}$ is formed, the presence of H_2O_2 becomes almost inevitable. Hydrogen peroxide is then converted into water and oxygen by the catalase enzyme. However, further reactions may lead to the formation of hydroxyl radicals ($OH^{\bullet}$), especially in the presence of metal ions through the Fenton or Haber–Weiss reactions (Fig. **1**). Hydroxyl radicals are extremely reactive, have a short half-life and will probably react with the first molecule they encounter. Another cellular source of ROS is xanthine oxidase, which catalyses the synthesis of uric acid from purines, forming $O_2^{\cdot-}$ and H_2O_2.

It thus appears that following the formation of $O_2^{\cdot-}$ a cascade of ROS production is initiated. Some of these ROS, especially H_2O_2, are key signalling molecules, while others appear to be extremely detrimental to biological systems, with effects that are dependent on the concentrations that are perceived by the cells [9].

Besides the enzymatic metabolism of oxygen-derived species, intracellular enzymes catalyse the production of RNS. Nitric oxide (NO), the major RNS, is formed endogenously from the oxidation of L-arginine to L-citrulline by a family of NADPH-dependent enzymes, the NO synthases (NOS) which exist in three isoforms: the neuronal NOS (nNOS) and the endothelial NOS (eNOS) forming the constitutive group, and the inducible NOS (iNOS). NO exists in different chemical forms (NO^- , $NO^{\bullet}$ and NO^+) and thus acts as an important oxidative biological signalling molecule in a large variety of physiological processes including neurotransmission, blood pressure regulation and immune mechanisms [10]. NO can also combine with $O_2^{\cdot-}$ to form peroxynitrite ($ONOO^-$), a highly reactive oxidant. Under physiological conditions, the constitutive NOS expressed in specific tissues produce NO for the cellular needs. The constitutive NOS are expressed in the inner ear by the hair cells and sensory neurons, and they produce small amounts of NO, which

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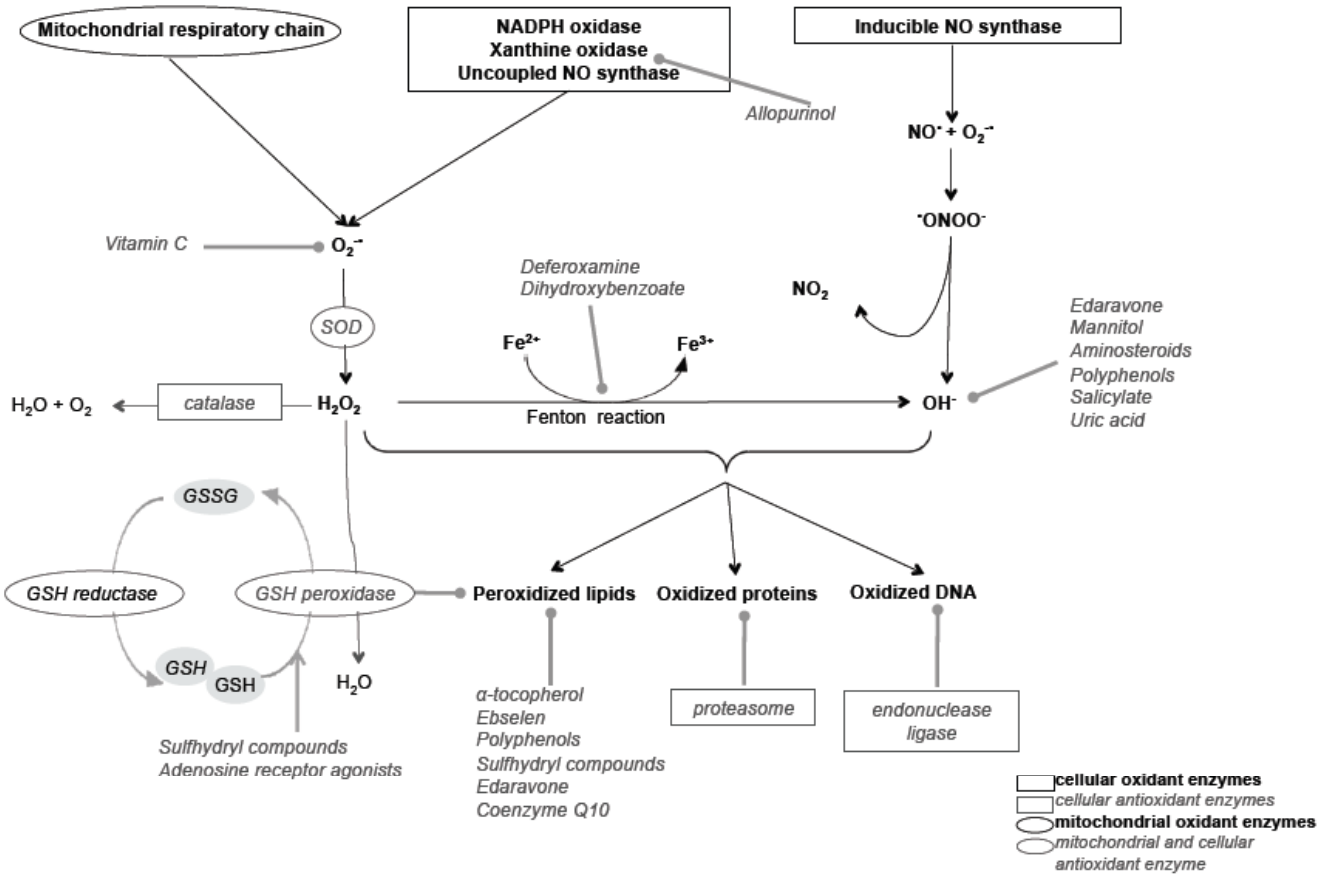

Fig. (1). Major pathways of cellular ROS/RNS generation and detoxification. Antioxidant elements are represented in *italics* characters. After the production of the superoxide anion ($O_2^{-\bullet}$), the superoxide dismutase (SOD) enzyme converts it to hydrogen peroxide (H_2O_2). Hydrogen peroxide (H_2O_2) is then converted into water by the catalase enzyme or by the glutathione peroxidase. Blunted grey arrows represent an inhibitory effect against oxidative stress by antioxidant enzymes or by antioxidant compounds. Oxidation of glutathione by the glutathione peroxidase enzyme allows electron transfer from ROS. Reduced glutathione (GSH) can be regenerated from oxidized glutathione (GSSG) by the glutathione reductase enzyme. NO produced by the inducible NO synthase can combine with $O_2^{-\bullet}$ to form peroxynitrite ($ONOO^-$), a highly reactive oxidant. Production of ROS leads to the generation of peroxidized lipids, oxidized proteins and oxidized DNA.

plays a role in blood flow regulation and neurotransmission [11-14]. Inhibition of the constitutive NOS leads to hearing impairment in gerbils [15]. By contrast, iNOS is not detected in the inner ear under physiological conditions, but can be virtually synthesised by any cell type when stimulated, and produces large amounts of NO. Indeed, iNOS expression and inadequate high amounts of NO are detected in the inner ear under pathological conditions, such as following the inoculation of lipopolysaccharide [16,17], the administration of cisplatin [18,19], aminoglycosides [20,21], or after noise exposure [22,23]. At the cellular level, immunoreactivity to iNOS under pathological conditions was found in the spiral ligament, modiolus, spiral limbus, supporting cells, nerve fibers and spiral ganglion cells neurons [14,16,19]. Direct evidence of NO production was also observed in the stria vascularis and hair cells following excitotoxicity [12,24].

To counteract oxidative and nitrosative insults, cells have developed two important defence mechanisms: a thiol reducing buffer and enzymatic systems, such as SOD, catalase and glutathione peroxidase. Thiol reducing buffer consists of small proteins, such as glutathione (GSH) or thioredoxin ($TRX\text{-}(SH)_2$) with redox active sulfhydryl existing under oxidized or reduced forms. Oxidation of glutathione or TRX by glutathione or thioredoxin peroxidase, respectively, allows electron transfer from ROS and thus results in radical neutralisation. Glutathione or thioredoxin reductase rebuilts the intracellular stock of reduced glutathione or reduced thioredoxin, using the coenzyme NADPH.

As intracellular second messengers, ROS/RNS can activate many downstream signalling molecules, including mitogen-activated protein kinases (MAPK), protein tyrosine phosphatases, and protein tyrosine kinases, to ultimately interrupt gene expression [25,26]. Alternatively, ROS/RNS might change gene expression by targeting and modifying the activity of transcription factors. Finally, ROS and RNS can react directly with lipids, DNA and RNA bases, metal cofactors, and proteins. Some of these pathways are clearly linked to enhanced survival, while others are associated with cell death. When the amount of oxidant compounds exceeds the physiological level, damages are initiated. Under pathological conditions, ROS may be produced by intracellular organelles, cell membrane or extracellular reactions. This ROS accumulation induces, *via* the activation of c-Jun-N-terminal kinases (JNK) and p38MAPK, the release of cyto-

chrome c from the mitochondria, and thus the activation of caspase-8, -9, and -3 (intrinsic pathway of apoptosis) [5]. By triggering the formation of autophagosomes and autolysosomes, ROS generated by the mitochondria also play a pivotal role in the autophagic cell death [27-29].

2. ROS/RNS IN OTOTOXICITY

ROS/RNS play a pivotal role in ototoxicity. Recent studies suggested that ROS/RNS are even a key factor in aging and presbycusis [30-36]. This review focuses on the cochlear injuries induced by aminoglycosides, cisplatin, excessive noise and aging. In the cochlea, all cell types do not share the same vulnerability to ROS/RNS injury. Outer hair cells seem to be most susceptible to free radical damage at the base of the cochlea while supporting cells have considerably more survival capacity than hair cells [37]. This should be explained by different patterns of protein expression among cochlear cell types. Indeed, it has been shown that glutathione levels are higher in apical outer hair cells than those at the base [37]. Moreover, NOX3, responsible for superoxide production, is specifically expressed in hair cells and in spiral ganglion neurons [7].

2.1. Aminoglycosides

Aminoglycosides are polycationic compounds largely used in clinical practice, especially in multiresistant Gram negative infections and tuberculosis. The ototoxic effects of aminoglycosides have been suggested to be due to intracellular cytotoxic effects depending upon their entry into the hair cells [38]. The mechanisms by which systemically administered aminoglycosides enter the cochlear fluids and tissues remain poorly understood. Recent studies have shown that aminoglycosides are electrically attracted to the negative-charged apical portion of the hair cells and enter by (1) permeating the mechanoelectrical transduction channels at the tips of stereocilia *in vitro* [39] or (2) apical endocytosis *via* a yet unknown receptor [40]. Indeed, systemically administered gentamicin rapidly enters marginal cells of the stria vascularis and hair cells in mice [41], suggesting that these drugs penetrate into the endolymphatic scala media of the inner ear, as hypothesised from cochlear perfusion experiments [42,43].

Once inside the cell, aminoglycosides induce the generation of ROS, a central player in the molecular pathway of ototoxicity [44-46]. Aminoglycosides are considered to be redox inactive compounds, and therefore a conversion to a redox-active form is necessary to induce ROS formation. Indeed, the generation of ROS involves the formation of an aminoglycoside-iron complex, which catalyses the oxidation of unsaturated fatty acids located into the inner leaflet of the plasma membrane [47-49]. In the absence of iron, arachidonic acid enriched in phosphoinositides can serve as an electron donor [47,50]. Aminoglycosides interfere with phosphoinositides metabolism, particularly phosphatidylinositol-biphosphate (PIP2), by binding to their polar head [51]. This binding induces sequestration of PIP2 and therefore inhibits PIP2-dependent processes [52], including the reduction of phosphatidylinositol-trisphosphate (PIP3) formation and the inhibition of the survival activities of the PIP3/Akt signalling pathway [53].

Aside from ROS formation, aminoglycosides are also known to directly modulate the activity of enzymes involved in ROS metabolism. They inhibit the antioxidant activity of catalase or activate iNOS producing NO [21]. Nitric oxide has been suggested to be involved in hair cell death induced by gentamicin application [54]. Aminoglycosides also indirectly promote the activation of NOX leading to the production of superoxide. Kanamycin treatment in mice activates Rac1, a member of the family of small Rho GTPases, and promotes the formation of the Rac1 and NOX complex, essential for the activation of NOX [55].

ROS subsequently activate apoptotic or necrotic intracellular pathways [56]. They promote the opening of the mitochondrial permeability pore and activate the JNK pathway leading to hair cell apoptosis [57,58]. The inhibition of JNK pathway rescues hair cells injured by aminoglycosides, both *in vitro* and *in vivo* [57,58].

Direct evidence for ROS accumulation in the outer hair cells after aminoglycoside exposure was first demonstrated by Hirose and colleagues [59] and further confirmed recently [60]. The degree of aminoglycoside-induced outer hair cell death increases along a baso-apical gradient both *in vivo* and *in vitro* [37,61]. This increased vulnerability of basal outer hair cells may be due to an intrinsic susceptibility to free radicals that differs among the cochlear cell population [37,44]. Iron chelators and free radical scavengers have been widely used for protection against the aminoglycoside challenge (Table **1**).

Hair cell injury and loss is followed by a retraction of the peripheral processes of the auditory nerve [62] and, later, by a gradual loss of spiral ganglion neurons [63,64]. Following aminoglycoside-induced hair cell injury, there is a progressive loss of spiral ganglion neurons as a consequence of loss of hair cell-derived neurotrophic support [65-67]. Neurotrophin withdrawal increases ROS production in neuronal cell lines [68,69] and in spiral ganglion neurons in culture [70]. The interaction of ROS and free radicals with membrane phospholipids creates lipid peroxidation products that act as mediators of apoptosis.

2.2. Cisplatin

Cisplatin is a platinum-containing compound widely used in oncology, especially in small cell lung cancer, lymphoma and ovarian cancer [71,72]. Cisplatin forms a monohydrated complex inside the malignant cell and cross-links DNA. This binding interferes with DNA replication by inducing production of DNA adducts, accumulation of which leads to malignant cell death [73]. Its clinical use is limited due to the induced nephro- and ototoxicity. In the cochlea, cisplatin administration is especially targeting outer hair cells in the basal turn of the organ of Corti, cells of the stria vascularis and the spiral ganglion neurons [45].

Some studies have demonstrated the direct cytotoxic mechanisms of cisplatin, including DNA damage, mitochondrial dysfunction, and the formation of ROS [74]. In the adult inner ear, where the cells are mainly post-mitotic, cisplatin toxicity is independent of DNA damage. Therefore, $O_2^{\cdot-}$ production by cisplatin may be the central mechanism of its ototoxicity. Indeed, the generation of ROS in the cochlea

Table 1. Major Antioxidant Molecules Used for Protecting Against *In Vivo* Aminoglycoside-Induced Ototoxicity

Type of antioxidant	Antioxidant molecule	Ototoxic drug	Animal	References
Enzymes	adenoviral vectors for overexpression of catalase and SOD	kanamycin + ethacrynic acid	guinea pig	[135]
Enzyme inhibitor	L-NAME (iNOs inhibitor)	gentamicin	guinea pig	[141]
Vitamin	alpha tocopherol (vitamin E)	gentamicin	guinea pig	[184,186,189]
Polyphenols	flavanoid fraction from *Drynaria fortunei*	gentamicin	guinea pig	[195]
	ginkgo biloba extract	gentamicin	guinea pig	[211]
	tanshinone	kanamycin	mouse	[212]
Iron chelators	deferoxamine	gentamicin	guinea pig	[154,159]
		neomycin	guinea pig	[156]
	deferoxamine, dihydroxybenzoate	kamamycin, streptomycin	guinea pig	[157]
	dihydroxybenzoate	gentamicin	guinea pig	[154,155,158]
		kanamycin	mouse	[213]
Sulfhydryl (thiol) compounds	N-acetyl-L-cysteine	gentamicin	human	[179]
	amifostine	kanamycin	guinea pig	[214]
	glutathione	gentamicin	guinea pig	[176,215]
	lipoic acid	amikacin	guinea pig	[177]
	D-methionine	amikacin	guinea pig	[164]
		gentamicin	guinea pig	[178]
	methylthiobenzoate, amifostine	gentamicin	guinea pig	[154]
	sodium thiosulfate	gentamicin	mouse	[216]
Other antioxidants	mannitol	gentamicin	guinea pig	[154,155,157]
		kamamycin, streptomycin	guinea pig	[157]
	ebselen	gentamicin	guinea pig	[141]
	edaravone	tobramycin	rat	[217]
	salicylate	gentamicin	guinea pig	[190]
		gentamicin	human	[198]

SOD = superoxide dismutase.

was demonstrated in guinea pig cochlear explants incubated with cisplatin [75] and in cultures of rat organ of Corti exposed to cisplatin [76]. Cisplatin-treated animals were also found to have increased immunoreactivities for 4-hydroxynonenal, a lipid peroxidation product, and for peroxynitrite in the outer hair cells and spiral ganglion neurons [77]. The increase in ROS generation could be due to depletion of thiol reducing buffers and antioxidant enzymes and/or direct activation of ROS generating systems. Indeed, cisplatin administration in rats is followed by a significant depletion of cochlear glutathione concentration, and a significant decrease in the cochlear SOD, catalase, glutathione peroxidase and glutathione reductase activities [78]. Moreover, cisplatin increases the expression of NOX3 mRNA in the spiral ganglion [7]. Following exposure to cisplatin, NOX3-dependent superoxide production is markedly enhanced and leads to the formation of hydrogen peroxide [79]. Down-regulation of NOX3 protects the hair cells and spiral ganglion neurons against cisplatin ototoxicity [80]. More recently, other NOX isoforms, NOX1 and NOX4, have also been shown to play a critical role in cisplatin-induced cochlear injury [81]. Suppression of NOX1 and NOX4 expression by siRNA transfection markedly abolished ROS generation and cytotoxicity induced by cisplatin. Xanthine oxidase activity also increases in the cochlea after systemic administration of cisplatin [82,83]. Inhibition of xanthine oxidase by allopurinol provides protection to the cochlea and the kidney after cisplatin challenge [84]. Concomitantly, an over-expression of iNOS is observed in the cochlea after cisplatin challenge [85] and, subsequently, leads to nitrosative stress. Finally, excessive ROS generated by cisplatin could overwhelm the antioxidant defence mechanisms within the cochlea, activating the pro-apoptotic pathway, which leads to outer hair cell death [86,87].

Thioredoxin reductase 1 (TxR1) is also a target for cisplatin in the cochlea [88]. This enzyme belongs to the large family of selenoproteins acting as major redox regulatory

agents and antioxidants [89]. In the presence of cisplatin, TxR1 highly reactive selenocysteine residue becomes compromised resulting in the inhibition of its reducing activity. Moreover, TxR1 is converted into a potential pro-oxidant killer [90].

Increased ROS triggers mitochondrial release of cytochrome c, through activation of the pro-apoptotic Bcl-2 family proteins. Subsequent apoptosis is then mediated by the activation of pro-caspase-9 and -3 [87]. More recently, it has been shown that ROS produced by cisplatin promotes activation of the transient receptor potential vanilloid 1 (TRVP1), which contributes to further increases in Ca^{2+} influx into the cell and finally to apoptosis of cells expressing these receptors, such as the outer hair cells or spiral ganglion neurons [80].

2.3. Noise Trauma

Exposure to excessive noise is the major avoidable cause of permanent hearing impairment worldwide, and is defined as an important public health priority by the World Health Organization [91]. Occupational noise is a major problem in the aging population and in developing countries. The incidence of noise-induced hearing loss in children and young adults is increasing especially with the exposure to portable music players [92]. Permanent hearing loss is due to the destruction of cochlear hair cells or damage to their mechanosensory hair bundles [93].

Loud sound causes a dramatic change in cochlear blood flow, including increased vascular permeability, capillary vasoconstriction, and blood stagnation in strial capillaries [94,95]. This renders the hair cells relatively anoxic and thus secondarily damaged. Noise exposure is also followed by an intense metabolic activity, due to over-stimulation [96,97]. Increasing evidence suggests that ROS are important role in noise-induced cochlear damage: increased levels of superoxide anion [98,99], hydroxyl radical [100], and RNS [22] are observed in the cochlea after intense noise exposure. In the organ of Corti, immunostaining for nitrotyrosine, a marker of RNS formation, shifts from supporting cells to outer hair cells following noise exposure [101]. In addition, one of the best established endogenous "fingerprints" of ROS action is the peroxidation of polyunsaturated fatty acids. Markers of lipid peroxidation have been demonstrated in the hair cells, supporting cells, spiral ganglion neurons and stria vascularis after noise trauma [101-103]. At the enzymatic level, noise exposure increases NOX activity in the cochlea and targeted deletion of SOD increases the susceptibility to acoustic injury, highlighting the importance of superoxide anion in noise-induced damage [104,105]. However, otoprotection by SOD remains controversial [106].

Thereafter, ROS accumulation initiates a complex cascade of biochemical processes that includes the activation of JNK and p38MAPK, the release of cytochrome c from the mitochondria and the activation of procaspase-8, -9 and -3 (intrinsic pathway of apoptosis). A caspase-independent pathway involving the endonuclease G translocation to the nucleus after noise trauma has also been reported [107,108]. Additionally, *in vitro* models of mechanical trauma showed an increased intracellular Ca^{2+} level [109,110]. The spreading of calcium wave may trigger an excessive release of glutamate in the cochlear efferent pathways, leading to excitotoxicity [111].

2.4. Presbycusis

Presbycusis is an extremely complex, multifactorial process, implying high frequency hearing loss concomitantly with physical signs of ageing [112]. Several molecular cascades have been implicated in age-related hearing loss, and the current consensus is that oxidative stress is one of its core mechanisms [30-36]. Indeed, genes that protect against oxidative stress are involved in development of age-related hearing loss [113-118]. Studies of the aging cochlea showed a decrease of antioxidant defenses such as glutathione level in the auditory nerve [119] or antioxidant enzymes in the organ of Corti and spiral ganglion neurons [112,120,121]. Significant loss of hair cells and spiral ganglion neurons has been observed in mice lacking SOD [122-124], and markers of oxidative and nitrosative stress are present in the organ of Corti and spiral ganglion of ageing mice [120]. In addition, there is a systematic degeneration of marginal and intermediate cells of the stria vascularis [125]. Strial intermediate cells normally produce melanin pigment, which has the ability to bind cations and metals to scavenge free radicals [126,127]. Indeed, cochlear melanin supports strial marginal cell survival and prevent age-related hearing loss [128,129]. Oral supplementation with the antioxidant lecithin (activating enzymes such as SOD) may prevent age-related hearing loss in rats [130]. However, overexpression of SOD in mice does not confer a resistance to the onset of presbycusis [131], and antioxidant agents have not been shown to counter auditory ageing [125]. Therefore, other signalling pathways should be implicated in presbycusis.

3. OTOPROTECTION AGAINST ROS/RNS

Oxidative damage may result from overproduction and/or lack of clearance of ROS/RNS by the scavenging mechanisms. Therefore, three major strategies avoiding oxidative stress in the inner ear can be developed: 1/ ROS detoxification by antioxidant enzymes, 2/ ROS interception by oxidant scavengers or 3/. inhibition of the downstream signalling pathways of ROS. Antioxidant systems can be divided into two groups: non-enzymatic and enzymatic. Enzymatic defences comprise agents that catalytically remove ROS, such as SOD, catalase, or glutathione peroxidase. Non-enzymatic antioxidants include intra- or extra-cellular low molecular weight compounds. They can be further classified into directly acting antioxidants (e.g. scavengers and chain breaking antioxidants) and indirectly acting antioxidants (e.g. chelating agents). Both groups of antioxidants have been widely used in otoprotective strategies.

3.1. Intracellular Antioxidant Enzymes

In humans, the importance of antioxidant enzymes machinery is illustrated by a large inter-individual vulnerability to ototoxic drugs. Clinicians have observed that chemotherapy-induced ototoxicity is associated with a polymorphism of glutathione-S-transferase (GST). Presence of both GST-P1G alleles and/or absence of functional GST-M1 protect against cisplatin-induced hearing impairment [132]. In

Table 2. Main Antioxidant Molecules Protecting Against *In Vivo* Platinum Anti-Cancer Agent-Induced Ototoxicity

Type of antioxidant	Antioxidant molecule	Ototoxic drug	Animal	References
Enzymes modulators	NOX3 SiRNA	cisplatin	rat	[218]
	Adenosine receptor agonists	cisplatin	chinchilla	[137]
	allopurinol (xanthine oxidase inhibitor)	cisplatin	rat	[84]
	aminoguanidine (iNOS inhibitor)	cisplatin	rat	[148,219]
	L-NAME (iNOS inhibitor)	cisplatin	guinea pig	[147]
Vitamins	alpha-tocopherol (vitamin E, trolox)	cisplatin	guinea pig	[183,185,187]
			rat	[188]
Polyphenols	CAPE (caffeic acid phenethyl ester)	cisplatin	rat	[83]
	ginkgo biloba extract	cisplatin	rat	[220]
Sulfhydryl (thiol) compounds	N-acetyl-L-cysteine	cisplatin	guinea pig	[166]
			rat	[167]
	amifostin	cisplatin	hamster	[221]
	diethyldithiocarbamate	cisplatin	rat	[142,222]
			hamster	[223,224]
	erdosteine	cisplatin	rat	[82]
	glutathione ester	cisplatin	rat	[225]
	lipoic acid	carboplatin	rat	[226]
		cisplatin	rat	[78,80,142]
	D-methionine	cisplatin	chinchilla	[162]
			guinea pig	[163]
			rat	[161,164,227]
	methylthiobenzoate	cisplatin	rat	[142,228]
	tiopronin	cisplatin	guinea pig	[187,189]
			rat	[229]
	sodium thiosulfate	cisplatin	guinea pig	[163,230-233]
			hamster	[223,224]
		carboplatin	human	[168]
	thiourea	cisplatin	guinea pig	[234]
Others antioxidants	ebselen	cisplatin	rat	[84,142]
	salicylate	cisplatin	rat	[191,192]
		cisplatin	guinea pig	[194]
	lazaroids	cisplatin	guinea pig	[235]
	dexamethasone	cisplatin	guinea pig	[236]
		cisplatin	mouse	[237]
	etanercept (anti-TNFα)	cisplatin	mouse	[81]
		cisplatin	rat	[238]

mammals, differential levels of expression of SOD or glutathione peroxidase may explain the unequal vulnerability of hair cells to aminoglycosides [37,119,133]. Therefore, genetic and pharmacologic manipulation of antioxidant enzyme levels could trigger powerful otoprotection against drug- or noise-induced hearing loss (Tables **1-3**).

Genetic Interventions by Antioxidant Enzymes

Experimental studies aimed at reducing oxidative stress by manipulating levels of antioxidant enzymes have shown encouraging results. SOD knockout mice have a greater susceptibility to noise trauma [104,134]. On the contrary, transgenic mice over-expressing SOD are protected against

Table 3. Antioxidant Molecules Shown to be Successful Against Noise-Induced Hearing Loss.

Type of antioxidant	Antioxidant molecule	Animal	References
Enzymes	SOD-PEG, CuZn-SOD	rat	[136]
		guinea pig	[239]
Enzymes modulators	adenosine receptor agonist (up-regulating antioxidant enzymes)	chinchilla	[138,139]
		rat	[240]
	allopurinol (xanthine oxidase inhibitor)	guinea pig	[239,241]
		rat	[136]
Vitamin	trolox	guinea pig	[242]
Iron chelators	deferoxamine	guinea pig	[151]
Sulfhydryl (thiol) compounds	N-acetyl-L-cysteine	rat	[182]
		guinea pig	[150]
		chinchilla	[243]
	glutathione	guinea pig	[244]
	D-methionine	chinchillas	[164]
	oxothiazolidine carboxylate	guinea pig	[245]
Other antioxidants	coenzyme Q10	guinea pig	[246]
	mannitol	guinea pig	[151]
	ebselen	guinea pig	[144,145]
	salicylate	mouse	[247]
		guinea pig	[242,243]
	tempol	guinea pig	[248]
	BN82270	guinea pig	[249]

kanamycin-induced hearing loss [133]. Adenoviral vectors-mediated over-expression of catalase and SOD administered into the cochlea of guinea pigs protect hair cells from aminoglycoside-induced toxicity [135]. Finally, siRNA directed against NOX3 decrease cisplatin-induced ototoxicity, both *in vitro* and *in vivo* [80].

Pharmacological Modulators of Antioxidant/Oxidant Enzymes

Activators and inhibitors of antioxidant or oxidant enzymes, respectively, have been widely used in otoprotective strategies. Allopurinol, a xanthine oxidase inhibitor, provides protection after noise trauma [136] or cisplatin challenge in the cochlea [84]. Similarly, a long acting SOD covalently linked to polyethylene glycol (PEG-SOD) preserves hearing threshold in rats exposed to noise injury [136]. Adenosine receptor agonists, which increase the activity of cochlear glutathione peroxidase and SOD, are effective in protection against cisplatin- [137] or noise-induced hearing loss [138,139].

Ebselen is a lipid-soluble seleno-organic compound that has been widely used as an antioxidant in experimental models, assuming that it acts *via* a GSH peroxidase like-mechanism [140]. In the cochlea, ebselen is reported to protect against damages induced by gentamicin [141], cisplatin [74,76,142,143], or noise trauma [144,145].

The NOS inhibitors, such as L-N(omega)-Nitroarginine methyl ester (L-NAME) or aminoguanidine, attenuate hearing threshold shifts and cochlear hair cell loss following cisplatin- [146-148], aminoglycosides- [149], or noise-induced injury [150]. Whether these drugs act through NOS inhibition remains to be established. Indeed, treatment of rats with aminoguanidine reduced cisplatin-induced ototoxicity but did not cause any reduction of NO production. Thus, aminoguanidine might act as a free-radical scavenger rather than an iNOS inhibitor [148].

3.2. Oxidant Scavengers in Otoprotection

Intra- and extra-cellular low molecular weight oxidant scavengers are fascinating molecules with a high therapeutic potential. As ROS/RNS plays a pivotal role in ototoxicity, these scavengers have been tested as otoprotective agents.

Metal Chelators

The condition of oxidative stress generated after ototoxic drug administration or acoustic trauma is accompanied, to a varying degree, by a dyshomeostasis of metal ions, including the redox-active transition metals: iron and copper [151-153]. Therefore, iron or copper chelation therapy could be a promising approach to prevent ototoxicity. Indeed, administration of the iron chelators, deferoxamine or 2,3-dihydroxybenzoate, protects hearing function and cochlear hair cells of guinea pigs treated by aminoglycosides [154-159]. Cochlear damage from noise trauma is also attenuated in the presence of deferoxamine [151].

Thiol-Containing Compounds

Low molecular weight thiol compounds, such as sodium thiosulphate, diethyldithiocarbamate, or L- or D-methionine, are electrophilic and can act directly as free radical scavengers. In addition, these thiol compounds are reported to induce cysteine uptake, thereby increasing the synthesis of intracellular glutathione [45,87].

Protection of the inner ear from cisplatin-induced damage has been tested with several thiol compounds (Table **2**), which may also act, in that particular case, as a sulphur containing nucleophile and thus protect sulphur containing enzymes and proteins. Glutathione supplementation prevents cisplatin-induced ototoxicity in guinea pigs [160]. Systemic or local inner ear delivery of D-methionine provides effective otoprotection after cisplatin treatment in rodents [161-164]. Systemic administration of N-acetyl-L-cysteine allows the preservation of hearing threshold in cisplatin-treated rats [165]. Trans-tympanic application of N-acetyl-L-cysteine prevents cisplatin-induced obliteration of distortion product oto-acoustic emissions in guinea pigs [166]. Recent study has demonstrated a greater efficacy of the association of two thiol compounds, N-acetyl-L-cysteine and sodium thiosulfate, against cisplatin-induced ototoxicity, both *in vitro* and *in vivo*. The protection provided by this association has been observed at the molecular, cellular, histological and functional levels [167]. Doolittle and colleagues have shown that sodium thiosulfate is effective in protecting against carboplatin-induced hearing loss in humans with malignant brain tumours [168]. Taken together, these studies have demonstrated that thiol compounds provide a good level of otoprotection against cisplatin damage to the cochlea. However, concerns about diminishing the oncological effects of cisplatin have limited the clinical use of thiol compounds [169-171]. Indeed, sodium thiosulfate, while providing a good level of protection against cisplatin damage to the cochlea, has been shown to also compromise its anti-tumour activity [169,172,173]. Protection from cisplatin-induced ototoxicity by systemic delivery of thiol compounds can be explained by a decreased systemic exposure to the platinum drug, and it therefore may cause a lowered antineoplastic activity [174]. Indeed, L-Methionine is efficient, either systemically or locally *via* the round window membrane, to protect hair cells from the structural damage induced by cisplatin [175]. However, systemic administration of L-Methionine results in a significant decrease in the antitumour activity of cisplatin, while local delivery does not interfere with its antitumour efficacy. These data indicated that the local administration should be the preferred method in otoprotection.

Thiol compounds also act as otoprotective drugs in aminoglycosides-induced ototoxicity [164,176-180]. N-acetyl-L-cysteine was the first radical scavenger used against kanamycin-induced ototoxicity [181]. It has been recently used in humans to ameliorate gentamicin-induced ototoxicity [179].

Similarly, hearing threshold shifts are diminished and hair cells are protected by N-acetyl-L-cysteine in rats exposed to noise trauma [182]. Recent data have also demonstrated that the administration of D-methionine rescues hearing function assessed by auditory brainstem responses from permanent noise-induced hearing loss when initiated one hour after noise exposure [164].

Other Antioxidant Molecules

A protective effect against drug-induced toxicity was also provided by broad spectrum ROS scavengers, such as vitamin E [183-189], salicylate [190-194] and antioxidant herbal extracts containing flavonoids *in vivo* [195] and *in vitro* [196,197].

Clinical Trials

Too few clinical trials have been conducted to allow any firm conclusions to be drawn about the efficacy and the safety of antioxidant therapy. Salicylate and N-acetyl-L-cysteine have been shown to decrease gentamicin-induced hearing loss in humans [179,198]. Sodium thiosulfate administered four hours after platinum treatment significantly decreased the time of hearing loss development and the rate of hearing loss in patients with malignant brain tumours [168]. Additional well-designed trials examining the benefit of antioxidant therapy are needed for cisplatin- or noise-induced ototoxicity.

3.3. Molecules Targeting the Downstream Signalling Pathway of ROS

Beneath the otoprotective agents that directly scavenge ROS, alternative strategies have been considered. One such strategy is to target the downstream signalling pathway of ROS/RNS. Indeed, noise exposure or administration of ototoxic drugs generates ROS, which in turn activates JNK and p38MAPK [199-201].

JNKs are potent effectors of apoptosis of oxidative stress-damaged cells in the inner ear following a trauma. The phosphorylation of c-Jun, a component of the transcription factor AP-1, is a central event in JNK-mediated apoptosis of oxidative stress-damaged auditory hair cells and neurons [202]. Inhibition of JNK prevents neomycin-induced cochlear hair cell death *in vitro* [203,204]. Moreover, inhibition of JNK protects against aminoglycoside-induced hearing loss and hair cell death in guinea pigs, when administered by local cochlear perfusion [58,205] or by subcutaneous injections [57]. Inhibition of JNK has also been shown to attenuate noise-induced hearing loss [203], but not the cisplatin-induced hearing loss [206].

The p38 MAPK pathway is also activated following ototoxic insult [207], and therefore constitute a promising target. Indeed, blockade of p38MAPK prevents gentamicin-induced hair cell loss *in vitro* [207,208], and noise-induced hearing loss *in vivo* [209].

4. CONCLUSIONS AND FUTURE DIRECTIONS

In the medical community, increasing attention is being given to prevention based on health measures such as diet, exercise, mental state and avoidance of drug abuse [210]. Because of the mechanisms of ototoxicity, the use of antioxidant therapy in inner ear toxicity is particularly relevant. These treatments could be used as soon as an ototoxic therapy is needed. However, there is currently no FDA-approved drug to prevent or treat ototoxin- or noise-induced hearing

loss. There is a need for a randomised controlled trial to establish with certainty not only the benefit of antioxidant therapy in human subjects but also the dose and the route of administration.

ABBREVIATIONS

GSH = glutathione

GSSG = oxidized glutathione

GST = glutathione-S-transferase

JNK = c-Jun-N-terminal kinases

L-NAME = L-N(omega)-Nitroarginine methyl ester

MAPK = mitogen-activated protein kinases

NADPH = nicotinamide adenine dinucleotide phosphate

NOS = nitric oxide synthase

iNOS = inducible NOS

nNOS = neuronal NOS

eNOS = endothelial NOS

NOX = NADPH oxidase

PEG = polyethylene glycol

PIP2 = phosphatidylinositol-biphosphate

RNS = reactive nitrogen species

ROS = reactive oxygen species

SOD = superoxide dismutase

TRVP1 = transient receptor potential vanilloid 1

TRX = thioredoxin

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