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Invited Review Article

Targeting dysregulation of redox homeostasis in noise-induced hearing loss: Oxidative stress and ROS signaling

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ABSTRACT

Hearing loss caused by exposure to recreational and occupational noise remains a worldwide disabling condition and dysregulation of redox homeostasis is the hallmark of cochlear damage induced by noise exposure. In this review we discuss the dual function of ROS to both promote cell damage (oxidative stress) and cell adaptive responses (ROS signaling) in the cochlea undergoing a stressful condition such as noise exposure. We focus on animal models of noise-induced hearing loss (NIHL) and on the function of exogenous antioxidants to maintaining a physiological role of ROS signaling by distinguishing the effect of exogenous "direct" antioxidants (i.e. CoQ_{10}, NAC), that react with ROS to decrease oxidative stress, from the exogenous "indirect" antioxidants (i.e. nutraceutics and phenolic compounds) that can activate cellular redox enzymes through the Keap1-Nrf2-ARE pathway. The anti-inflammatory properties of Nrf2 signaling are discussed in relation to the ROS/inflammation interplay in noise exposure. Unveiling the mechanisms of ROS regulating redox-associated signaling pathways is essential in providing relevant targets for innovative and effective therapeutic strategies against NIHL.

1. Introduction

1.1. Overview on the organ of hearing

The hearing organ cochlea, sited in the inner ear, houses a sophisticated machinery responsible for the detection of air-borne sound vibrations and the conversion from mechanical energy into electrical signals [for extensive description see ref 1]. The machinery comprehends a multitude of highly specialized cell types (the organ of Corti), a complex arrangement of inner ear fluid-filled compartments and a vascular organ, the *stria vascularis* that provides maintenance of ionic equilibrium of fluid compartments and metabolic support for the organ of Corti (Fig. 1A). Within the sensory epithelium of the organ of Corti two major classes of sensory cells (inner -IHC and outer -OHC hair cells) are arranged in rows (Fig. 1A_1) [2,3]. IHCs, disposed in a single row, are the mechano-transduction cells that allow for the detection of sound and the transmission, through synaptic connection with auditory nerve afferent fibers, of information about the acoustic environment to the central auditory system for its representation and recognition. The more numerous OHCs, known as the cochlear amplifier, arranged in three rows, are involved in the complex process known as cochlear amplification, contributing to the high sensitivity, wide dynamic range, and sharp frequency selectivity of our hearing. Thus, sound vibrations, amplified by OHCs are detected by IHCs [4] and changes in their cell polarization are coupled to regulated release of glutamate neurotransmitter at the synapses between the IHCs (synaptic ribbons: a rounded or linear structure to which neurotransmitter vesicles are tethered) and the terminals of auditory nerve fibers that form synapses around the IHC's basolateral membrane and connect the sensory cells to spiral ganglion neurons (Fig. 1A, F-J).

1.2. Overview on noise-induced hearing loss (NIHL)

Both sensory hair cells are extremely fragile, vulnerable and in mammals, upon injury (i.e. noise exposure, ototoxic medications, aging), cannot regenerate [1,5]; permanent sensorineural hearing loss ensues leading to detrimental communication impairments and adverse health effects. Among the different damaging factors, a leading cause of hearing impairment in industrialized countries, defined as noise-induced hearing loss (NIHL), is caused by exposure to recreational, environmental and occupational noise. As reported by the World Health Organization, NIHL prevalence is of about 16% in adult population

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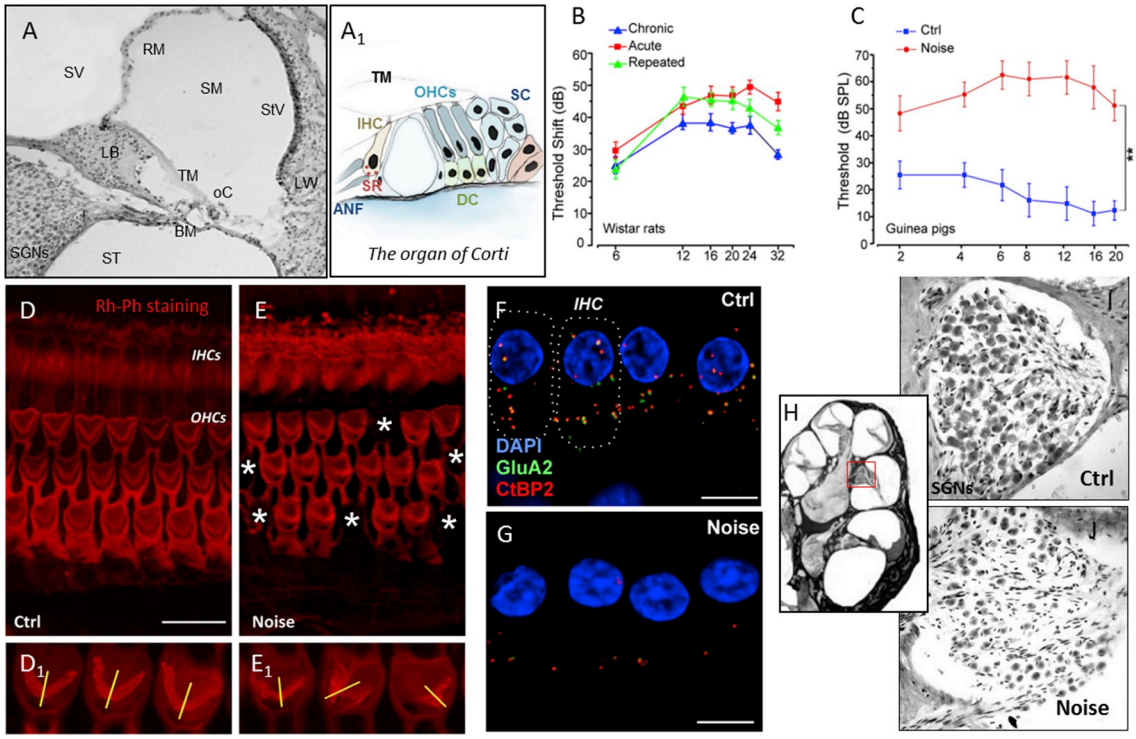

Fig. 1. Functional evaluation and characteristic morphological features of NIHL.
A: Representative cochlear cryosection stained with Hematoxylin and Eosin, showing the principal cochlear structures (SV: Scala Vestibuli; SM: Scala media; ST: Scala Timpani; RM: Reissner membrane; BM; basilar membrane; TM: tectorial membrane; LB: limbus; SGNs: spiral ganglion neurons; oC: organ of Corti; StV: *stria vascularis*; LW: lateral wall). A_1: Schematic representation of the organ of Corti cellular organization (ANF: afferent nerve fibers; SR: synaptic ribbons; IHC: inner hair cell; OHCs: outer hair cells; DC: Deiter's cells; SC: supporting cells). B-C: *Auditory evaluations.* A similar hearing impairment of about 40 dB SPL (ABR recording evaluation) in rats and guinea pigs exposed to different paradigms of noise. B: Threshold shift values (means ± SEM) in Wistar rats after repeated (100 dB SPL, 60 min, 10 consecutive days) and chronic (98 dB SPL, 60 min, 5 days/week, 3 consecutive weeks) and acute (120 dB SPL, 60 min) noise exposures. C: Auditory thresholds (means ± SEM) in control and noise-exposed (acute acoustic trauma) guinea pigs. Asterisks indicate significant differences between groups (**p < 0,01). D–E: *Noise-induced outer hair cell loss*. Representative images of surface preparations of the organ of Corti stained with Rhodamine-Phalloidin (Rh-Ph) showing the typical distribution of OHC three rows and IHC one row in control specimens (D). The dark spots indicated by asterisks in E show OHC loss after repeated noise exposure. The typical V shape of stereocilia and the orientation of hair bundles (indicated by yellow lines) is represented in D_1 (higher magnification), while noise causes the disorganization of OHC hair bundle, characterized by disruption of the V shape and disorientation of the hair bundles, mainly in the middle-basal turn, as shown in E_1. F–G: *Signs of synaptopathy*. Noise-induced synaptopathy and representative images of synaptic ribbons (red, anti-CtBP2; green, anti-GluA2) in IHC area of cochlear middle-basal turn. Outlines of selected IHCs are indicated (dashed lines in F). Images show juxtaposed pre-synaptic ribbons and post-synaptic receptor and the lack of both in noise-exposed animals (G) with respect to controls (F). H–J: *Noise-induced SGN degeneration.* Representative longitudinal section of the cochlea indicating (red box) middle turn SGN area (H). Noise exposure decreased SGN density with both neuronal soma and auditory fiber damage (J). Control condition (I). Scale bar: A, 100 μm; D-E, F-G, 10 μm; I-J, 50 μm. Adapted from *Fetoni* et al. *2013, Maulucci* et al. *2014, Paciello* et al., *2018.* (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

worldwide [6–9] with significant differences among regions reaching 21% in the developing world and 35% in the countries of East Europe [10]. NIHL is usually characterized not only by the elevation in hearing threshold [11,12], speech perception and auditory processing disorders [13–16] but it is also associated to symptoms such as the phantom sound tinnitus, the increased sensitivity to loud sounds hyperacusis [17–19] and to a range of non-auditory health effects (sleep disorders, impairment of cognitive performance, cardiovascular diseases etc.) [8,20–22]. The incidence of NIHL and its linked detrimental health effects are bound to increase by the interaction with accelerated age-related hearing loss given the high incidence of noise exposure and the aging of our society [23–27]. In addition, the susceptibility to noise differs among individuals due to the combination of genetic and environmental factors. Genetic evidence points to a link between mutations found in both mitochondrial genes and endogenous antioxidant defense-related genes and individual susceptibility to NIHL [28–31]. Genetic variations in knockout mice (Cadherin 23, GJB2, $p66^{shc}$ and heat shock proteins genes) increase or decrease NIHL and presbycusis susceptibility [26,32–34]. For example, defective *Ahl* allele of the gene encoding for cadherin 23 in C57BL/6 (B6) mouse strain is considered a model for aging and NIHL susceptibility [26]. $p66^{shc}$, is a proapoptotic protein involved in ROS production in mitochondria [35–37] and knock-out mice lacking $p66^{shc}$ gene are resistant to age-related diseases and oxidative stress injuries [38,39]. Screening of single nucleotide polymorphisms of different oxidative stress genes known to play a functional role in the inner ear is a promising approach [40–42].

1.3. NIHL features

NIHL has obvious public health significance and a relevant socio-economic impact however, currently, no effective pharmacologic agents are approved by Food and Drug Administration (FDA) to diminish NIHL. Development of an efficacious treatment has been hampered by the complex array of cellular and molecular pathways involved in NIHL. Most evidence has been obtained through morphological, electrophysiological and immunohistochemical studies in several animal models of noise exposure. NIHL features are characterized by hearing threshold elevation as detected by auditory brain stem measurements (ABR) (Fig. 1B and C), disruption of stereociliary structure and permanent loss of the auditory sensory cells in the cochlea [29,43–48] particularly the prominent loss of OHCs (Fig. 1D and E) while loss of IHCs is limited [49–51]. Damage mostly occurs in the high

frequency regions of the cochlea, more basal along the cochlear spiral than the frequency place of the original noise exposure [52] and hair cell susceptibility follows a base-to-apex pattern [53,54]. Evidence on loss of IHC synaptic ribbons and synaptic contacts (Fig. 1F and G), extensive spiral ganglion neuron (SGN) degeneration (Fig. 1H–J) [46,52] and damage of *stria vascularis* have been demonstrated [55,56]. In addition, the effects of noise can be exacerbated by exposure to chemicals such as organic solvents [56,57] and by certain ototoxic drugs [58–60].

Remarkably, as several studies have shown [52,61,62], the impact of hearing loss might be underestimated by the evidence for "hidden hearing loss" and disorders of IHC synapses (synaptopathy) are associated to poor speech recognition observed in patients exposed to noise [14,63]. In NIHL animal models high level noise exposure causes damage to and loss of hair cells, resulting in permanent decrements in hearing sensitivity, dramatic swelling of a proportion of the afferent buttons and degeneration of the afferent nerve fibers [64] caused, at the synaptic connection between IHCs and auditory nerve fibers, by noise-induced robust Ca^{2+} influx and glutamate excitotoxicity [65–67]. However, also milder noise exposures, that result in a temporary hearing impairment, still cause permanent damage to the afferent nerve fibers in the absence of any loss of hair cells [52,68]. Some studies suggest that loss of most terminals is permanent [52,68,69], other evidence indicates that some synapses can be repaired [70–72]. Indeed, the synaptic connections between IHCs and the afferent auditory fiber terminals may be impaired at the pre-synaptic side (presence of multiple ribbons, and decrease in the number of ribbon-attached vesicles) [63,70,71] and, in a recent study [73], it has been shown that pre-synaptic regions adjacent to damaged terminals have ultrastructural changes that may affect their function (altered recycling of synaptic vesicles from membrane cisterns). The synaptopathic mechanism is well established in other conditions of auditory neuropathy (i.e. genetic auditory neuropathies) [74,75].

1.4. Damaging mediators of NIHL

A widely accepted mediator of cochlear noise-induced damage is the excess of free radical oxygen species (ROS) formation [50,76–81]. In the cochlea, the role of ROS in damage initiation and progression has been supported by the generation of ROS in cochlear tissues observed immediately after exposure to damaging levels of noise, well before morphological signs of damage are detected and persisting for 7–10 days after exposure [76,82]. ROS-induced lipid peroxidation products, as observed after noise exposure, can lead to apoptosis and vasoactive lipid peroxidation products (i.e isoprostanes) reduce cochlear blood flow [83]. Noise-induced ischemia and subsequent reperfusion potentiates further the generation of ROS [84–86]. ROS generation in the cochlea can also lead to production of pro-inflammatory cytokines that can further produce damage [87–89]. Similar mechanisms have been reported to occur in drug-induced hearing loss [56,59,90] and aging [25,26,91–95].

As to the source of ROS, a primary generator is the mitochondrion which generates ROS as byproducts of metabolism [96,97]. Indeed a major and widely accepted mechanism underlying NIHL is mitochondrial ROS formation due to noise-induced intense metabolic activity in the cochlea [50,80,81]. After noise exposure, mitochondrial aerobic respiration increases and the large quantities of ROS are not efficiently neutralized. ROS egress into the cytosol as detected in cochlear structures by increased superoxide and lipid peroxidation production [27]. This eventually leads to IHC and largely to OHC death through either apoptosis or necrosis [50,84–86,98,99]. Mitochondrial ROS are reported to provide the feedback regulation after metabolic excess [100] the regulation of the hypoxia-inducible factor 1 during low oxygen conditions [101–103], the regulation of autophagy [104–107] and of the inflammatory response [108–110].

Additional contributors to ROS generation in NIHL involve intracellular calcium (Ca^{2+}) homeostasis and NAD(P)H oxidases. Free Ca^{2+} has been found to increase in cochlear HCs immediately after exposure to damaging noise [111]. Ca^{2+} release from the endoplasmic reticulum and/or entry from extracellular fluid leads to loss of mitochondrial membrane potential, increased membrane permeability and release of ROS from mitochondria [12]. Elevated calcium may not only induce cytoplasmic ROS accumulation, but may also trigger apoptotic and necrotic cell death pathways independent of ROS [112]. Remarkably, intracellular Ca^{2+} may be a contributing factor making OHCs in the high frequency region of the cochlea, most vulnerable to environmental assault [113] and a Ca^{2+} tonotopy gradient has been exhaustively reviewed recently [114]. Ca^{2+} influx and voltage-gated Ca^{2+} channel complex have been associated to IHC synaptopathy and excitocitosis [74,115–117]. The NAD(P)H oxidases [118–120], membrane-bound proteins that transfer electrons across the plasma membrane to molecular oxygen, may also be contributory factors to ROS generation in NIHL [121,122] as reduced permanent hearing loss has been reported following intracochlear treatment with a NAD(P)H oxidase inhibitor under conditions of noise-induced cell stress [123].

1.5. Dysregulation of redox status: oxidative stress and ROS signaling

ROS-induced oxidative stress and disruption of redox status have been reported to play a relevant role in several systems [124–127] and in cochlear damage [27,50,81,84,93,127]. Damaging ROS comprise the free radicals superoxide ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}HO$), singlet oxygen ($^1O_2$) and the non radical hydrogen peroxide (H_2O_2) [128–130]. The oxidant species $O_2^{\bullet -}$, generated by the one-electron reduction of $O_2^{\bullet -}$ in the mitochondrial electron transport chain [105,131] and through cytosolic NAD(P)H oxidases is rapidly converted to H_2O_2 by the enzymatic activity of the enzymes dismutases (SODs: mitochondrial SOD2, cytosolic SOD1) giving raise to the extremely reactive $^{\bullet}HO$, (H_2O_2 reaction with metal cations Fe^{2+} or Cu^{+} via the Fenton reaction) which indiscriminately oxidizes lipids, proteins, and DNA, resulting in damage or genomic instability [132]. To prevent the buildup of H_2O_2 and the toxicity of $^{\bullet}HO$, potent antioxidant systems exist to spatially and temporally regulate intracellular ROS levels. H_2O_2 is converted to water by the enzymatic activity of several antioxidants including peroxiredoxins (PRXs), glutathione peroxidases (GPXs), and catalase [133 and references within]. Within the mitochondrion the potent antioxidant enzymes rely on NAD(P)H as source of reducing equivalents. NAD(P)H is used to maintain the GPX or PRX antioxidant systems via the glutathione (GR) and thioredoxin (TR) reductase enzymes [134]. Furthermore, under normal conditions, adequate intracellular ROS levels are essential to regulate cellular homeostasis [127] and the generation of ROS [135,136] through cell signaling pathways [108,109,127,137]. Indeed, there have been numerous reports highlighting the importance of ROS-dependent signaling in a variety of systems [138] and the release of ROS has evolved as a method of communication between mitochondrial function and other cellular processes to maintain homeostasis and promote adaptation to stress [127,139].

Studies over the past two decades have been addressed to pharmacological strategies to decrease the impact of NIHL by using molecules able to neutralize the excess of noise-induced ROS. However, ROS promote cell adaptation as well [109,132,139] and relevant therapeutic benefit can be obtained by activating specific ROS signaling pathways that regulate stress-protective responses [140 and references within].

1.6. Objective

In this review article, we will distinguish the effect of exogenous antioxidants (named "direct"), that react with ROS to decrease noise-induced oxidative stress, from the exogenous antioxidants (named "indirect") that activate cellular redox enzymes [140]. The term of direct and indirect antioxidants was introduced in the mid-1990s by FDA in order to distinguish between antioxidants (“direct”), which are redox

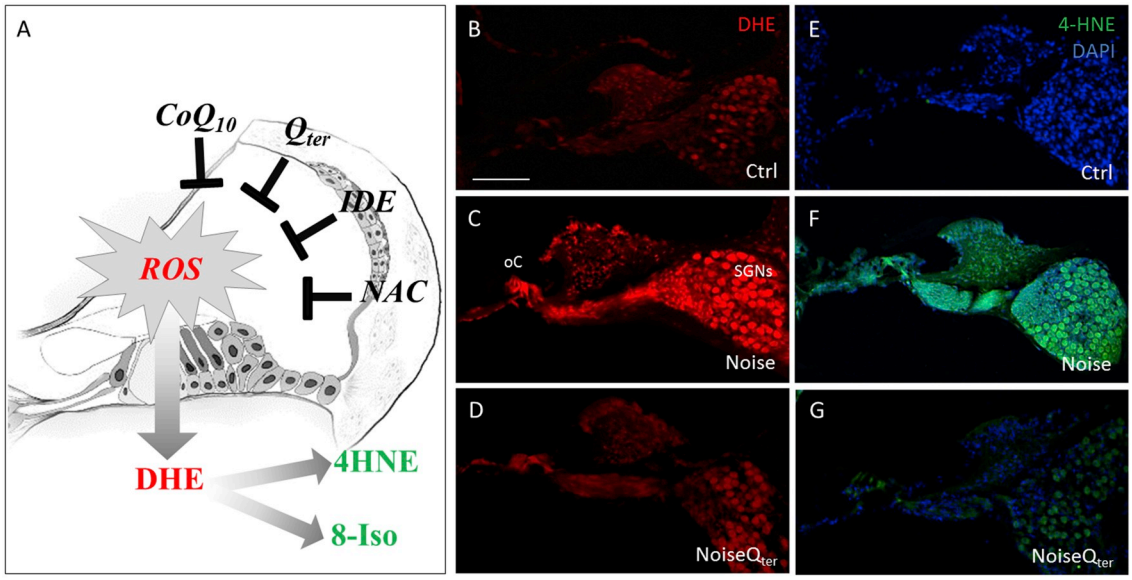

Fig. 2. Oxidative stress and adaptive responses induced by exogenous "direct" antioxidants.
A: *Schematic representation showing the effect of exogenous antioxidants against cochlear oxidative stress*. Noise induces free radical accumulation (ROS) in the organ of Corti (DHE assay for superoxide anion detection), and rise of plasma membrane peroxidation (4-HNE and 8-Isoprostane markers). Supplementation of "direct" exogenous antioxidants attenuates cochlear oxidative damage by scavenging superoxide and decreasing peroxidative damage. CoQ_{10}: Coenzyme Q_{10}; Q_{ter}: Coenzyme Q_{ter} IDE: Idebenone; NAC: N-acetyl-cisteine; VIT E: Vitamin E. B–G: *Noise-induced oxidative stress*. B–D: Representative confocal images of cochlear cryosections showing superoxide amount (DHE staining, red fluorescence). Fluorescence is faint in control tissue (B), increased in Noise samples (C), and decreased in $NoiseQ_{ter}$ group (D), indicating that the antioxidant supplementation with Q_{ter} attenuates superoxide accumulation after noise exposure. E–G: *Noise-induced lipid peroxidation*. Representative confocal images of cochlear cryosections immuno-labeled with lipid peroxidation marker 4-HNE (green fluorescence) and double stained with DAPI (blue fluorescence). 4-HNE expression increases in noise-exposed animals (F) both in the organ of Corti and spiral ganglion neurons, control sample (E). Q_{ter} administration attenuates lipid peroxidative buildup in all cochlear structures (G). oC: organ of Corti, SGNs: Spiral ganglion neurons. Scale bar: 100 μm. Adapted from *Fetoni* et al. *2013*. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

active, short-lived, are sacrificed in the process of their antioxidant actions and need to be replenished or regenerated, and may evoke even prooxidant effects and antioxidants ("indirect") that may or may not be redox active, nutrients that are precursors of coenzymes that are involved in oxidative reactions but do not have direct antioxidant activities [126,141]. "Indirect" antioxidants activate the ROS signaling pathway Kelch-like ECH-associated protein 1 (Keap1)-nuclear factor erythroid 2-related factor 2 (Nrf2)- antioxidant response element (ARE) resulting in transcriptional induction of a battery of cytoprotective proteins (also known as phase 2 enzymes) that control ROS intracellular homeostasis. We will thus consider the activation of the ROS signaling Keap1-Nrf2-ARE complex that constitutes the best characterized transcription factor with an oxidant/electrophile-sensing capability [142–146]. Nrf2 is considered a ROS receptor in mammals, regulates environmental and xenobiotic stress-protective responses and has also been known to attenuate inflammation [147,148]. In addition, a growing body of evidence strongly suggests an etiological role of oxidative stress-associated inflammation and cell death in the development of many human diseases [149,150]. Cumulative evidence in the cochlea has indicated an interrelation between ROS and inflammation in noise trauma, cisplatin ototoxicity and auditory problems usually associated with aging [12,151–155]. Noise exposure has been shown to up-regulate cochlear production of cytokines [88,151] and of tumor necrosis factor alpha (TNFα) [156], both of which have been observed after ROS generation in the cochlea [89,157]. Generation of these proinflammatory mediators can occur via activation of the nuclear factor kappa B (NF-κB) signaling cascade, leading to cytokine production [158]. ROS interact with the NF-κB signaling pathway in many ways [138,159,160] and the transcription of NF-κB dependent genes has been shown to influence the levels of ROS in the cell and, in turn, the levels of NF-κB activity are also influenced by the levels of ROS [159,162,163]. NF-κB proteins are a family of transcription factors that are of central importance in inflammation [164,165]. Furthermore the anti-inflammatory activity of Nrf2 has been suggested to relay on modulation of redox metabolism or crosstalk with NF-κB [159,163].

Unveiling the mechanisms of ROS regulating redox-associated signaling pathways is essential in providing relevant targets in order to develop innovative and effective therapeutic strategies against NIHL.

2. Oxidative stress and exogenous "direct" antioxidants

A plethora of experimental studies have demonstrated in the last two decades that the magnitude of hearing loss induced by noise can be modulated by pharmacological intervention [80,166,167]. Just to mention a few, tested antioxidants include glutathione (GSH) [168], allopurinol [169], evadarone [170], D-methionine [171,172], P-PIA and monoesthelester [173], ebselen [174], resveratrol [175], vitamin C [176], acetyl-*l*-carnitine (ALCAR) [177], N-acetyl, L-cysteine (L-NAC) [178], Vitamin E and alpha lipoic acid [179]. Ebselen reduced the extent of noise-induced cell injury preventing acute and repeated noise exposures in guinea pigs and rats, acting as mimic of GPX [174,180,181]. ALCAR has been used to improve mitochondrial membrane energetics in NIHL [177], treatment with either ALCAR or NAC, significantly reduced the permanent hearing loss in chinchillas exposed to impulse noise [182]. NAC and hydroxylated alpha-phenyl-tert-butylnitrone (4-OHPBN), a nitrone-based spin trapping agent of $^{\bullet}HO$ and $O_2^{\bullet -}$, decreased hearing impairment and OHC loss in a model of acute pure tone noise exposure (105 dB SPL) [183]. NAC is probably the most studied molecule in terms of its efficacy on reducing noise trauma under a variety of conditions, animal models and dosages [178,182,184–187]. NAC, similarly to GSH pro-drugs, directly scavenges H_2O_2 and hydrogen radicals and is a major contributor to the maintenance of cellular GSH acting as substrate for its synthesis [188]. GSH repletion also adds substrate for GPX enzyme and other GSH-related enzymes promoting the direct, intrinsic antioxidant activity of GSH-ROS scavenging. However the effectiveness of direct GSH

supplementation is controversial mainly for its poorer bioavailability as compared to NAC [140].

We used small molecules that react with ROS to decrease oxidative stress and in the attempt to reproduce the human acoustic trauma, detrimental for speech frequencies, we focused our studies on a frequency-specific NIHL model in guinea pigs, rats and mice by using a pure tone noise exposure (6 kHz centered noise exposure in guinea pigs and 10 kHz in rats and mice). Oxidative stress was analyzed in the organ of Corti, spiral ganglion and *stria vascularis* by measuring $O_2^{\bullet -}$ production (Dihydroethidium-DHE assay) and lipid peroxidation (4-hydroxy-2-nonenal, 4-HNE, and isoprostanes). 4-HNE is one of the more sensitive and widely biomarkers of lipid peroxidation used *in vitro* and *in vivo* experimental models [189]. Morphological damage in the cochlea was verified by scanning electron microscopy (SEM), phalloidin (Fig. 1D and E), TUNEL assay and apoptosis markers. To address the mitochondrial respiratory chain we used molecules such as Coenzyme Q_{10} (CoQ_{10}), its analogs Idebenone and Q_{ter}, having the latter enhanced bioavailability with respect to the native form [46,51,190–192]. We also tested some direct scavengers such as alpha-tocopherol [191] and NAC [187] (Fig. 2A). Specifically, we studied the effectiveness of these molecules (systemic administration) using different protocols of noise exposure: acute exposure (120 dB SPL, 40/60 min), repeated exposure (100 dB SPL, 60 min, 10 consecutive days) and chronic exposure (98 dB SPL, 60 min, 5 days/week, 3 consecutive weeks), Thus, we implemented protocols of preventive, delayed and extended treatments reaching the conclusion that the antioxidant supplementation in a peritraumatic "therapeutic window" can attenuate significantly NIHL in all the different models of acoustic trauma [193–195]. As regards the effects of CoQ_{10} and its liposoluble analog, Q_{ter}, we demonstrated that Q_{ter} can attenuate auditory threshold elevation induced by noise (both in a rat and guinea pig model), targeting the redox imbalance in the cochlea (Fig. 2B–G) [46] and, consequently, attenuating cell death and apoptotic pathway activation in the organ of Corti [51]. Our results, although indirectly, suggest that the exogenous administered CoQ_{10} may be incorporated into mitochondria, where it may enhance electron transfer and ATP synthesis to neutralize ROS. The bioenergetic improvement due to enhanced electron transfer is certainly the major mechanism by which CoQ_{10} administration can counteract oxidative stress [196]. Interestingly, reduced oxidative stress was consistent with increased levels of endogenous quinone (CoQ_9) after the administration of Q_{ter} indicating that the exogenous quinone can exert a protective effect and scavenging activity as shown by the higher CoQ_9 levels (i.e. major form expressed in rats of endogenous quinones) in noise-Q_{ter} treated animals with respect to the noise controls when the noise exposure had ceased [see 46 for details]. Similar results were obtained by our group with idebenone, that showed protective effect in a guinea pig model of acute NIHL [190]. Idebenone can effectively scavenge a variety of free radical species, including peroxynitrite, peroxyl and tyrosyl radicals, and hypervalent states of hemoproteins [197].

These studies extend earlier findings in several important ways. In particular, our data on NIHL models provide evidence on oxidative stress in the cochlea: enhanced superoxide production and lipid peroxidation in hair cells and SGNs and demonstrate the oxidative status after noise exposure (Fig. 2C,F). A strong immunoreactivity for 4-HNE is detected in almost all OHCs in the damaged area in the first 24 h as reported after the acoustic trauma in guinea pigs [198]. Interestingly, an increasing level of free radical-induced lipid peroxidation is revealed in OHCs and SGNs during the first 18 h [198] up to 3–7 days after the exogenous insult [194,195]. The finding that free radical scavengers administered as long as 3 days post-noise exposure attenuate free radical formation, reduce sensory cell death (OHCs and SGNs), and reduce NIHL has been supported by administering Q_{ter} for days after noise exposure [46,51]. Interestingly, the local (transtympanic) and systemic routes of Q_{ter} administration showed a similar degree of hearing protection and decrease of the antioxidant stress biomarkers [192].

Moreover, our group investigated the consequences of acoustic trauma and cochlear deafferentation in the central acoustic system. We observed in the pyramidal neurons of auditory cortical areas decreased spine density [see 46 for details]. Namely, noise exposure induced oxidative stress damage in the sensory epithelium of the organ of Corti and degeneration of SGNs and the upward spread of cochlear oxidative damage appeared to cause plastic rearrangement in the pyramidal layers (layers 2/3 and 5/6) of the auditory cortex. The decrease of the peripheral oxidative imbalance by Q_{ter} antioxidant treatment, reversed the upward spread of the cochlear damage and the deafferentation consequences in the auditory cortex, specifically in the highly plastic neurons of the auditory cortex layer 2/3 [19,46,199].

Finally, even if antioxidants target the major pathway for cell death, the efficacy of any single antioxidant appears to be limited by several factors, including the limited access to cellular compartments, action against only a few forms of ROS, interference with redox-based signaling, or a tendency to throw innate ROS protections out of balance [24]. Thus therapies combining multiple antioxidants, or antioxidants plus other agents have been proposed. Some studies with diet supplementation based on the combination of beta-carotene, vitamins C and E, and magnesium [167,200] or with NAC plus 4-OHPBN or 4-OHPBN plus NAC plus ALCAR [183], provided evidence for a greater efficacy in attenuating NIHL. However we demonstrated that protective effects of idebenone and vitamin E were not additive implying that the two antioxidants may share competitive mechanisms [191]. The *in vivo* pro-oxidant/antioxidant activity of some direct exogenous antioxidants has been found to depend on their interaction with biological membranes and the other co-antioxidant molecules like vitamin C or E. Therefore, the balance between oxidant production and antioxidant protection is believed to be critical in maintaining healthy biological systems. Development of biomarkers of oxidative stress could be useful in disease monitoring, early detection and prevention for oxidative stress-associated human diseases including NIHL [201].

However there are discrepancies in the evaluation of potential protectants in different studies and, in translational medicine, systemic and chronically applied direct antioxidants may not necessarily have beneficial effects or even cause reductive stress or harm [7,140,202].

3. ROS signaling and adaptive responses induced by exogenous "indirect" antioxidants

It is acknowledged that despite the abundance of endogenous antioxidants, residual intracellular ROS remain to participate in signal transduction allowing for the co-localization of elevated H_2O_2 with numerous signaling components without risking oxidative damage to the rest of the cell [108,132,203,204]. The levels of hydrogen peroxide determine the physiological outcomes and by relating intensity of noise to the levels of ROS a working hypothesis is that very high quantities of ROS (severe noise) directly damage proteins, lipids, and nucleic acids, lower levels of ROS (moderate noise) function as signaling molecules to adapt to the stress and even lower levels of ROS (mild noise) are required for normal cell homeostasis [127]. In the cochlea, severe intensity of noise impacts on the extent of damage and not only the hair cells but the entire organ of Corti and cochlear architecture is disrupted, ROS signaling components are inefficient and cell death machinery is activated [50,98,205–208]. Whereas, ROS signaling may be crucial when the cochlea undergoes stimulations at moderate and even mild intensities [127]. The major mechanism by which cells increase their antioxidant adaptive responses is through activating the transcription factor Nrf2 [145,146,209,210].

3.1. Nrf2 –ARE pathways: activation of endogenous antioxidant enzymes

By testing the protective effects of a phenolic compound (Rosmarinic acid -RA) in a rat model of NIHL we analyzed the adaptive stress response of the Nrf2 signaling pathway in cochlear structures (organ of Corti, SGNs, *stria vascularis*) [195]. In our *in vivo* NIHL model,

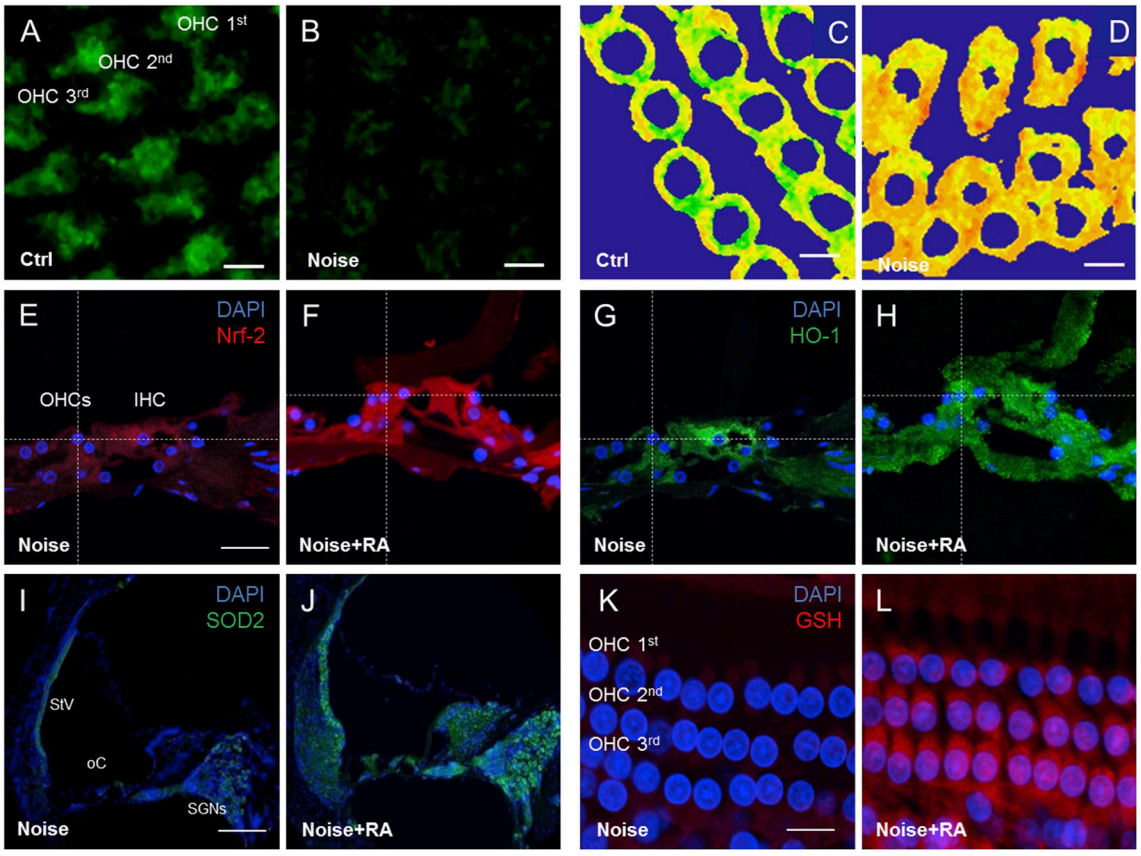

Fig. 3. Activation of the adaptive stress response through ROS signaling pathways.
A–D: *Noise induces NAD(P)H oxidation and decrease of plasma membrane fluidity.* A–B: Representative images of surface preparations of the organ of Corti showing NAD(P)H fluorescence in control OHCs (A) and noise exposure (B). Reduced NAD(P)H, molecule intrinsically fluorescent, is excited by two photons of near infra-red light, the NAD(P)H oxidized form (NAD(P) +) is not fluorescent. Fluorescence is absent in noise-exposed samples (B) indicating an alteration of the metabolic cellular state. C–D: OHC membrane fluidity images in control OHCs (C) and noise exposure (D). The cell membranes from fluid (green, C) become gradually more rigid (red, D) after noise exposure indicating membrane destructuration. E–L: *Increase of antioxidant enzymes through Nrf2 pathway activation.* E–H: Representative images of the organ of Corti double labeled with antibodies against Nrf2 (E,F) and HO-1 (G,H) and stained with DAPI. The antioxidant supplementation of the phenolic compound Rosmarinic acid (RA) potentiates Nrf2 nuclear translocation and increases HO-1 expression after noise exposure (F,H). I–J: Representative immunofluorescence labeling of cochlear cryosections for SOD2 (green fluorescence) double-stained with DAPI (blue fluorescence) showing RA-induced increase of SOD endogenous antioxidant response after noise exposure in all cochlear structures. K–L: Representative images of surface preparation of the organ of Corti with double labeling for GSH (red fluorescence) and DAPI (blue fluorescence). Noise overstimulation induces a slight increase in GSH expression (K) further up-regulated by RA administration (L). Scale bars: A-D, 8 μm; E-H, 30 μm; I-J 100 μm; K-L, 20 μm. OHC: Outer hair cell, IHC: Inner hair cell, oC: organ of Corti, SGNs: Spiral ganglion neurons, StV: *Stria vascularis*. Adapted from *Fetoni* et al. *2015, Maulucci* et al. *2014.* (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

noise increased superoxide anion generation, which in turn injured neural cells, as demonstrated by the detection of lipid peroxidation byproduct 4-HNE. In response to noise-induced damage, cells in the organ of Corti and SGN activated the adaptive stress response through the ROS signaling pathway: slight cytosolic up-regulation of Nrf2, heme oxygenase (HO-1) and activation of SODs as evidenced by fluorescence analysis (Fig. 3E,G,I). Despite this early attempt to combat cell damage, the endogenous antioxidant system failed to restore redox homeostasis and the defense activity was not sufficient to prevent cochlear damage. RA treatment increased remarkably the expression of Nrf2 both in the cytoplasm and in the nucleus of cells in the organ of Corti (see Fig. 3F). Nrf2 translocation paralleled, in the same slices, the up-regulation and expression of HO-1 in hair cells (Fig. 3H) and SGNs [195]. Thus, the Nrf2-induced up-regulation of HO-1 influenced the survival of the amplifying OHCs and the transmission of the primary afferent neurons to the central acoustic pathway. This Nrf2-induced up-regulation of HO-1 confirmed a previous *in vivo* observation in the guinea pig NIHL model on the importance of HO-1 adaptive response in otoprotection induced by another phenolic compound, ferulic acid [193]. The inducible isoform of HO-1, the microsomal enzyme deputed to heme catabolism, exerts its antioxidant function by removing a pro-oxidant molecule, heme, while simultaneously producing metabolites which are endowed with unique protective characteristics, that is, carbon monoxide and biliverdin, which is further converted to bilirubin by biliverdin reductase [211–213]. Biliverdin and bilirubin possess remarkable antioxidant properties and participate in the protection of cells and tissues against oxidative stress [214]. Iron is also released during heme degradation by HO-1, and its increased intracellular levels result in up-regulation of ferritin, an iron-storing protein that participates in the cytoprotective machinery engaged by HO-1 to combat stress conditions [213,215]. Furthermore, the Nrf2-induced enhancement of the adaptive stress response was also linked to increased generation of GSH and to its scavenger activity [195]. GSH is the most abundant antioxidant in cells and tissues and plays a primary role in protection against oxidative stress [216]. The increase of GSH adducts was observed at the same time points as RA-induced increase of Nrf2 and HO-1. The auditory function at the mid-high frequencies was ameliorated and the loss of OHCs and IHCs damage by noise exposure at the basal/middle cochlear turn was limited. Consistent with our findings is a recent study on $Nrf2^{-/-}$ mice examined for susceptibility to NIHL [217]. These authors

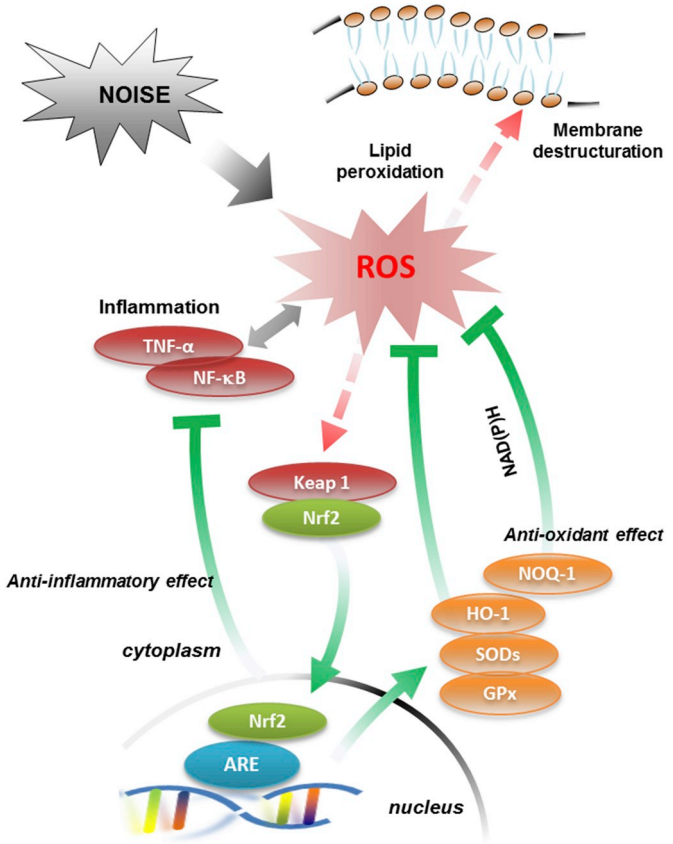

Fig. 4. ROS signaling pathways in the cochlea activated by noise insult. Schematic representation showing noise-induced ROS signaling in the cochlea and Nrf2-induced activation of cytoprotective proteins (SODs, HO-1, GPx, NAD(P)H) and anti-inflammatory pathways.

reported ABR threshold shifts at 7 days post-exposure significantly larger in $Nrf2^{-/-}$ mice than wild-type mice and treatment with CDDO-Im, a potent Nrf-2-activating drug, before but not after the noise exposure preserved the integrity of hair cells and improved post-exposure hearing levels in wild-type mice, but not in $Nrf2^{-/-}$ mice. Nrf2 is a master regulator of cell homeostasis that regulates the expression of antioxidant and cytoprotective genes that contain a specific enhancer sequence in their regulatory regions ARE. Under basal conditions, it is sequestered in the cytoplasm, where it is targeted for ubiquitin-mediated proteolysis, whereas after oxidative stress, Nrf2 is stabilized and translocated to the nucleus, where it activates the ARE-responsive genes [218–220]. These genes account for more than 1% of the human genome and include antioxidant genes, heme oxygenase-1 (HMOX1 coding HO-1) and nicotinamide adenine dinucleotide phosphate (NAD(P)H) quinone oxidoreductase-1 (NQO-1 coding NAD(P)H), gene encoding enzymes involved in GSH metabolism and others [145,146,210]. Nrf2 allows adaptation and survival under conditions of stress by regulating the gene expression of diverse networks of cytoprotective proteins, including antioxidant, anti-inflammatory, and detoxification enzymes as well as proteins that assist in the repair or removal of damaged macromolecules. Indeed, Nrf2 has a crucial role in the maintenance of cellular redox homeostasis by regulating the biosynthesis, utilization, and regeneration of GSH, thioredoxin and NAD(P)H and by controlling the production of reactive oxygen species by mitochondria and NAD(P)H oxidase [146]. As regards GSH, in addition to its biosynthesis, Nrf2 provides the means for the maintenance of glutathione in its reduced state by the coordinated transcriptional regulation of GSH reductase which reduces oxidized GSH using the reducing equivalents from NAD(P)H [109,221,222]. Nrf2 regulates transcriptionally the principal NAD(P)H-generating enzymes as well as the inducible gene expression of the cytosolic, microsomal, and mitochondrial forms of aldehyde dehydrogenase, which use NAD(P) as a cofactor, giving rise to NAD(P)H [146]. Compared to wild type animal, the total mitochondrial NADH pool is significantly increased in Keap1-KO and dramatically decreased in Nrf2-KO cells [223]. Reduced NAD(P)H is an intrinsically fluorescent molecule which may be exploited as a label-free method for monitoring the intracellular redox state of living cells and tissues and several advanced approaches have been developed with the introduction of scanning confocal microscopy (single photon −340 nm light in the UV spectrum and two photon near-infra red (NIR) light) and fluorescence lifetime imaging (FLIM) techniques [134,198,224,225]. We investigated by two photon excitation microscopy NAD(P)H reducing power in the distinct functional regions of the organ of Corti cells, primarily in the OHCs, by analyzing NAD(P)H spatial distribution and time evolution of its oxidation following noise exposure, together with the generation of lipid hydroperoxides and the organization of the plasma membrane by analyzing its fluidity [see 198 for details on methods and microscopy evaluations]. Lipid peroxidation disturbs the asymmetry of membrane lipids and may cause major changes in membrane characteristics including changes in fluidity [226]. Optimal fluidity is critical to OHC shape and function [227]. First following the acoustic stress, we observed, with respect to control values, a significant reduction of reduced NAD(P)H (increased NAD(P)H oxidation), indicative of changes of OHC redox state (Fig. 3A–B) [228,229]. The increase of NAD(P)H oxidation was followed by a rise of plasma membrane peroxidation and an equivalent decrease of fluidity (Fig. 3C–D). The trend of NAD(P)H decrease, peroxidation increase and fluidity decrease showed a close relationship and the correlation between lipid peroxidation and membrane fluidity was indicative of a cause/effect relationship. The decreases in membrane fluidity, secondary to lipid peroxidation [228,230], can lead to membrane destructuration and to modulation of the intermolecular interactions between the molecular motor prestin and lipids and/or the associated complex pattern of cytoskeletal elements and submembranous endoplasmic reticula [for further details see 198 and references within, 231,232]. Interestingly, we found that the drop of reduced NAD(P)H was characterized by a fast oxidation time (~0.36 h), before reaching a steady state. During this steady state, free radical accumulation led to a consequent rise of plasma membrane peroxidation (peroxidation time ~4.0 h). This in turn led to a membrane destructuration (destructuration time ~7.6 h). After the onset of membrane destructuration, triggered by lipid peroxidation, NAD(P)H decreased till exhaustion with a total oxidation time of ~9.4 h. These observations are of particular clinical interest since the development of an effective antioxidant intervention plan would increase the protection of OHCs from cell death [198].

3.2. ROS signaling/inflammation interplay

Several studies have demonstrated inflammatory responses in the cochlea following exposure to traumatic noise involving, in addition to oxidative stress, the up-regulation of pro-inflammatory mediators and rapid recruitment of inflammatory cells from the vascular system [88,151,152,157,233–235]. Also chronic environmental noise exposure in mice can induce cochlear damage and hearing loss via inflammatory processes [89]. Over the past few decades numerous studies have indicated that NF-κB is a key transcription factor driving inflammation and that TNFα, ROS, and NF-κB are inextricably tied together in inflammation, immunity, and cancer [163,236–238]. Although it is clear that ROS are crucial for NF-κB signaling downstream of TNFα [163,239], debate is ongoing over whether mitochondrial ROS are involved in NF-κB activation or inactivation [163]. Using the mitochondria-specific antioxidant MitoVit E it has been confirmed that mitochondrial ROS are important for NF-κB activation [240]. To our knowledge, it is not yet understood how mitochondrial ROS activate NF-κB in the cochlea, but it is assumed that ROS inactivate the

Table 1
Update and relevant researches on NIHL and ROS signaling.

TOPIC	MAIN AGENTS	MAIN FINDINGS	REFERENCES
Damage Mediators	**ROS**	- Noise-induced ROS production disrupts redox status, induces hypoxia/blood flow reduction, loss of HCs at cochlear midle/basal turn, loss of SGNs, *stria vascularis* degeneration. Mitochondria major source for oxidant production, central to metabolism, key roles in apoptosis, oxygen sensing during hypoxia, calcium homeostasis, autophagy. ROS activation of transcription factor Nrf2 is the major mechanism of antioxidant proteins increase.	[50,76,78,80–86,98–107,111–114,195,217]
	Ca^{++} Excitotoxicity	-Noise-induced Ca^{++} "flooding" leads to loss of mitochondrial membrane potential, increased membrane permeability, release of ROS from mitochondria and may trigger apoptotic and necrotic cell death pathways. -Noise induces "hidden hearing loss" due to Ca^{++} driven glutamate exitotoxicity, altered synaptic communication between inner hair cells and auditory nerve fibers.	[63–67,70,71,73–75,111,112,114–117]
	Inflammation	-Interrelation between ROS and inflammation in the cochlea after noise exposure associates NIHL to inflammation and production of cytokines: TNF-α, IL-1β and IL-6, produced in fibrocytes after noise exposure.	[12,88,151–156]
	NAD(P)H Oxidases	-NAD(P)H oxidases (NOX family) produce superoxide via a single electron reduction. Noise exposure influences NOX expression. NOX Inhibition is potentially a pathway for NIHL therapeutics.	[118–123]
ROS Signaling molecules and redox homeostasis	**Nrf2**	-Redox signaling maintain redox status through activation of Keap1-Nrf2-ARE complex that provides transcriptional induction of genes HMOX1 coding HO-1. NQO-1 coding NAD(P)H and genes involved in GPX metabolism (induction of cytoprotective proteins and redox homeostasis)	[142–148,195,210,217,219,220]
	HO-1	-HO-1 removes prooxidant molecule, heme, produces metabolites with protective characteristics (CO, biliverdin).	[193,211–215]
	NAD(P)H	-NAD(P)H is the ultimate reductant for ROS-catabolizing enzymes (i.e. GPX, PRX). Its oxidation following noise exposure induces, through lipid peroxidation, plasma membrane fluidity loss and OHC functional impairment.	[109,134,198,221,222,224,225,229]
	NFκ-B	-NF-κB is of central importance in inflammation. ROS interacts with NF-κB signaling pathways in many ways. Transcription of NF-κB dependent genes influences cellular ROS levels and levels of NF-κB activity are regulated by ROS levels. PPARγ direct negative interaction with NF-κB regulates inflammation.	[158–160,162,164,165,244,249]
Exogeneous Protective Molecules	**-"Direct" antioxidants: scavengers**	-Low-molecular-weight re-cycling antioxidants acting as scavengergs of hydroxyl radicals and superoxide anions (Vit E, Vit C, NAC), mimic of glutathione peroxidase (ebselen), substrate for GSH synthesis (NAC), mitochondrial membrane energetic improvement (ALCAR), enhanced electron transfer and ATP synthesis (CoQ_{10}, Q_{ter}, Idebenone).	[46,51,56,174,176,178,180,184,187,191,202]
	-"Indirect" antioxidants: activators of adaptive responses	-Noise-exposure activates adaptive stress response through slight cytosolic Nrf2, HO-1 and SOD up-regulation. Phenolic compounds (Rosmarinic, Ferulic acids) increased Nrf2 expression in cytoplasm and nucleus of organ of Corti and SGNs. Nrf2 translocation paralleled up-regulation and expression of HO-1 in hair cells. Nrf2 deficiency exacerbates NIHL.	[193,195,210,211,216,217]

phosphatases that regulate the activity of the kinases controlling NF-κB signaling. Such ROS-mediated phosphatase inhibition would lead to enhanced phosphorylation of IkB, triggering its degradation and permitting NF-κB activation [159,241]. Moreover Nrf2, essential for protection against oxidative damage as reported above, has well established anti-inflammatory properties and, contrary to the current hypothesis that Nrf2 represses inflammation as a secondary consequence of its upregulation of numerous antioxidant genes and elimination of ROS, Nrf2 inhibits the induction of proinflammatory cytokine gene transcription [242,243]. Indeed, recent evidence has suggested a mechanism of transcriptional repression of proinflammatory cytokines (TNFα, Interleukins (i.e. IL-1 and IL-6) and others) as reported, the binding of Nrf2 in close proximity of the IL-6 and IL-1β genes may imply that Nrf2 inhibits this transcription through direct DNA binding [242]. The molecular basis how Nrf2 elicits the transcriptional inhibition needs to be further elucidated.

We recently observed in a rat model of NIHL the attenuation of both the inflammatory markers NF-κB and IL-1β and the oxidative ones superoxide production and lipid peroxidation by using pioglitazone [244], an agonist of the peroxisome proliferator-activated receptor γ (PPARγ), that belongs to the nuclear hormone receptor superfamily of transcription factors [245–248]. PPARγ is able to regulate inflammatory processes in multiple organs by a direct interaction with NF-κB. NF-κB controls a vast number of genes involved in inflammation while PPARγ is able to regulate inflammation by a direct negative interaction with NF-κB [249]. Furthermore, antioxidant function of PPARγ has also been reported and several studies have suggested the existence of a regulation between Nrf2 and PPARγ pathways to reinforce the reciprocal expression [250–252]. In relation to the ROS signaling/inflammation interplay, it can be mentioned an additional possible signaling pathway based on our observation on $p66^{shc}$ deficient mice 24 h after acute noise exposure [39]. We reported that 129SvEv mice lacking the $p66^{shc}$ protein were resistant to the impairment of auditory function induced by noise in their $p66^{shc}$-proficient controls. Histochemical and biochemical signs of oxidative stress, inflammation and compromised cochlear blood flow were absent or attenuated in

p66-deficient mice. We proposed that such a functional defect resides in the establishment of $p66^{shc}$ and ROS-triggered endothelial dysfunction and compromised cochlear blood flow. $p66^{shc}$ in mitochondria, has been suggested to function as a redox enzyme possibly oxidizing cytochrome c [36,137]. However the molecular mechanism by which $p66^{shc}$ expression is increased in response to stress signals remains at the moment uncharacterized in the cochlea [137].

Altogether, the activation of the ROS signaling pathway Nrf2 in the noise-exposed animals operates adaptive stress responses through the gene-regulation of at least two antioxidant/detoxifying enzymatic systems: NAD(P)H quinone oxidoreductase-1 and HO-1 (Fig. 4). The anti-inflammatory properties of Nrf2 signaling are also well established however, a further insight and elucidation is needed as regards the molecular basis how Nrf2 regulates negatively the target genes that encode inflammatory cytokines and how Nrf2 interacts with the inflammation agents NF-κB and PPARγ pathways.

4. Concluding remarks

In this article, we have reviewed the role of ROS in the development of NIHL, the role of pharmacological targeting against oxidative stress trough the use of antioxidants, the role of Nrf2, a major ROS signaling cascade agent, and the potential cross-talks between ROS and NF-κB and between Nrf2 and PPARγ pathways (Table 1). Many studies have detailed a plethora of tests of various drugs and agents as potential therapeutic interventions in noise damage. Most studies have shown protective capacity against NIHL in animal research by addressing small molecules, that react with ROS non-enzymatically and that can be recycled or replenished giving them a ROS-buffering capacity. The timing of antioxidant treatment was shown to be crucial and the development of effective antioxidant intervention protocols should provide a peri-traumatic approach based on the evidence of a "therapeutic window" in which redox unbalance might be effectively counteracted. However, with the exception of a few studies in other pathologies, antioxidants acting as scavengers have almost always failed to show a significant effect in long-term clinical trials performed according to the criteria of evidence-based medicine. Only a few studies have analyzed in NIHL molecules that can interact with ROS signaling pathways to induce adaptive responses that could include induction and nuclear translocation of redox response elements such as Nrf2 however, promising findings indicate that, also in the cochlea, Nrf2 is the key regulator for the two important cytoprotective pathways, anti-oxidation and anti-inflammation. Thus, future studies should be designed to query the interaction between Nrf2 and other transcription factors (e.g. NF-κB and PPARγ), as well as Nrf2 effector molecules (e.g. NAD(P)H and HO-1), in order to better understand the mechanisms through which these genes induce adaptive responses against noise-induced ROS production and inflammation.

Understanding how oxidative stress and ROS signaling impact on NIHL in animals may provide therapeutic insights to counteract hearing loss in humans and the identification of novel disease strategies. The impact of noise exposure on the adolescents and young population is a further challenge in improving knowledge of mechanisms for a target therapeutic approach.

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