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Research paper

Noise-induced changes in expression levels of NADPH oxidases in the cochlea

Srdjan M. Vlajkovic [a,*], Shelly Ching-yu Lin [a], Ann Chi Yan Wong [c], Brad Wackrow [a], Peter R. Thorne [a,b]

[a] Department of Physiology, Faculty of Medical and Health Sciences, The University of Auckland, Private Bag 92019, Auckland 1142, New Zealand
[b] Discipline of Audiology, Faculty of Medical and Health Sciences, The University of Auckland, New Zealand
[c] Translational Neuroscience Facility, School of Medical Sciences, University of New South Wales, Sydney, Australia

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ABSTRACT

NADPH oxidases are enzymes that transport electrons across the plasma membrane and generate superoxide radical from molecular oxygen. The current study investigated the expression and distribution of NOX/DUOX members of the NADPH oxidase family (NOX1-5 and DUOX1-2) in the rat cochlea and their regulation in response to noise. Wistar rats (8–10 weeks) were exposed for 24 h to band noise (8–12 kHz) at moderate (100 dB) or traumatic (110 dB) sound pressure levels (SPL). Animals exposed to ambient noise (45–55 dB SPL) served as controls. Immunohistochemistry demonstrated predominant expression of all NOX/DUOX isoforms in the sensory and supporting cells of the organ of Corti, with very limited immunoexpression in the lateral wall tissues and spiral ganglion neurons. Noise exposure induced up-regulation of NOX1 and DUOX2 in the cochlea, whereas NOX3 was down-regulated. A significant reduction in the intensity of NOX3 immunolabeling was observed in the inner sulcus region of the cochlea after exposure to noise. Post-exposure inhibition of NADPH oxidases by Diphenyleneiodonium (DPI), a broadly selective NADPH oxidase inhibitor, mitigated noise-induced hearing loss. Conclusion: Noise-induced up-regulation of NOX1 and DUOX2 could be linked to cochlear injury. In contrast, down-regulation of NOX3 may represent an endogenous protective mechanism to reduce oxidative stress in the noise-exposed cochlea. Inhibition of NADPH oxidases is potentially a novel pathway for therapeutic management of noise-induced hearing loss.

1. Introduction

Hearing loss is the greatest cause of sensory disability (WHO), affecting up to 1 in 6 of the population. It is estimated that approximately 20% of the burden is generated from excessive noise exposure in occupational and leisure settings (Thorne et al., 2008). Sustained exposure to sound pressure levels >85 dBA or sudden exposure to impulse noise leads to irreversible damage to the sensory structures of the cochlea. Once damaged, the mammalian sensory hair cells do not regenerate, and the loss of hearing is permanent. Oxidative stress is considered a major contributing factor to noise-induced cochlear injury, along with glutamate excitotoxicity, inflammation, compromised blood flow and alteration of ion homeostasis. In addition, oxidative stress in the cochlea appears to be a common factor for hearing loss from noise, aminoglycoside antibiotics, ototoxic anticancer drugs and aging (Henderson et al., 2006). During noise exposure, the electron transport chain of the mitochondria uses large amounts of oxygen, which can then create large amounts of superoxide generated as an unwanted byproduct. Excessive production of superoxide can then generate higher levels of other reactive oxygen species (ROS) such as hydrogen peroxide and hydroxyl radical through Fenton reaction, or react with nitric oxide to generate reactive nitrogen species (RNS) such as peroxynitrite (Henderson et al., 2006). Free radicals are capable of damaging DNA, breaking down lipid and protein molecules, and triggering cell death, all of which can contribute to the hair cell lesion and loss of function seen after noise (Henderson et al., 2006). ROS can also trigger apoptosis by activating proapoptotic MAP kinase signalling pathways, such as SAPK/JNK, ERK1/2, and p38 (Irani, 2000). Noise exposure places especially high demands on the mitochondria of the outer hair cells to generate large amounts of energy through aerobic respiration, and this leads to their increased vulnerability to oxidative stress.

Abbreviations: ABR, auditory brainstem response; BSA, bovine serum albumin; DPI, Diphenyleneiodonium; DUOX, dual domain oxidase; EDTA, ethylendiamintetraacetate; NADPH, nicotinamide adenine dinucleotide phosphate; NDS, normal donkey serum; NIHL, noise-induced hearing loss; PBS, phosphate buffer saline; PFA, paraformaldehyde; RNS, reactive nitrogen species; ROI, region of interest; ROS, reactive oxygen species; RWM, round window membrane

* Corresponding author. Tel.: +64 9 3737599; fax: +64 9 3737499.
E-mail address: s.vlajkovic@auckland.ac.nz (S.M. Vlajkovic).

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Characterisation of free radical sources in the cochlea opens the way to new therapeutic approaches to mitigate hearing loss. The continued free radical formation for up to two weeks after experimental noise exposure (Yamashita et al., 2004) provides a window of opportunity to reduce cochlear injury in the post-exposure period. Two important strategies to ameliorate oxidative stress involve increasing cochlear antioxidant supplies and decreasing ROS production; both can substantially prevent hair cell damage and hearing loss. Antioxidants are molecules that scavenge ROS/RNS and convert them to less dangerous molecules. Antioxidants include glutathione, vitamins C and E, as well as enzymes such as superoxide dismutase, catalase, and various peroxidases. Antioxidant defences in the cochlea can be augmented by a variety of antioxidants, and glutathione-based strategies (ebselen, N-acetylcysteine, D-methionine) appear to be particularly effective (Le Prell et al., 2007; Oishi and Schacht, 2011). Partial protection against noise-induced hearing loss (NIHL) can be also achieved using dietary antioxidants, such as vitamins A, C and E, and magnesium (Oishi and Schacht, 2011), or a combination of salicylate and trolox (a synthetic analogue of vitamin E), which is also effective in the post-exposure period (Yamashita et al., 2005).

Mitochondria are not the only source of free radicals in the cochlea, and in this study we were aiming to identify other molecular mechanisms of ROS generation in the noise-exposed cochlea which could then be exploited in pursuit of novel therapeutic strategies for NIHL and other forms of acquired hearing loss caused by oxidative stress. The focus is on nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, the family of seven superoxide-generating enzymes (NOX1, NOX2, NOX3, NOX4, NOX5, DUOX1 and DUOX2) often playing a role in host defence through ROS-dependent bacterial killing (Krause, 2004; Bedard and Krause, 2007). In the central nervous system, NADPH oxidase activity is required for neuronal signalling, memory and central cardiovascular regulation, whilst overproduction of ROS by NADPH oxidases contributes to neurotoxicity, neurodegeneration and blood-barrier damage in experimental stroke (Infanger et al., 2006; Kahles et al., 2007). The aim of this study was to investigate the expression and distribution of NOX/DUOX members of the NADPH oxidase family in the rat cochlea as a possible source of free radicals, and to determine their contribution to noise-induced cochlear injury.

2. Materials and methods

2.1. Animals

Cochlear tissues were obtained from male adult (8–10 weeks old) Wistar rats sourced from the animal facility at the University of Auckland. Animals were euthanised using sodium pentobarbitone (100 mg/kg, i.p.) for tissue collection. From each animal, one cochlea was collected for mRNA extraction and the other for immunohistochemistry. All procedures in this study were approved by the University of Auckland Animal Ethics Committee and conformed to international guidelines for the ethical use of animals.

2.2. Isolation of cochlear mRNA and screening for NOX/DUOX transcripts by RT-PCR

Rat cochleae were removed, placed into sterile 0.1 M phosphate-buffered saline (PBS; pH 7.4, pre-chilled to 4 °C) and decapsulated under dissection microscope. The membranous labyrinth and modiolus were homogenised in cold lysis buffer (100 mM TRIS-HCl pH 8.0, 500 mM LiCl, 10 mM EDTA pH 8.0, 1% LiDS, 5 mM dithiothreitole) using a sterile Teflon pestle. Polyadenylated RNA (mRNA) was extracted using Dynabeads® mRNA DIRECT™ kit (Invitrogen). First-strand cDNA synthesis was carried out in a 20-μl reverse transcription (RT) reaction with random hexamers, dNTPs and Superscript III reverse transcriptase (Invitrogen). The complementary DNA was amplified by PCR with rat-specific primers for NADPH oxidase isoforms (Table 1) designed using OligoPerfect™ (Invitrogen). Negative control without reverse transcriptase was included in each PCR run. RT-PCR with a 40 cycle profile was performed as follows: 94 °C denaturation (1 min), 60 °C annealing (1.5 min), 72 °C extension (2 min) steps using PTC-100™ Programmable Thermal Controller (MJ Research Inc., Waltham, M.A., USA). PCR amplicons were separated by agarose gel electrophoresis, and visualised using SYBR safe DNA gel stain (Invitrogen). PCR product was purified by PureLink™ PCR Purification Kit (Invitrogen) and the identity of the amplicon was confirmed by DNA sequencing (Centre for Genomics & Proteomics, School of Biological Sciences, the University of Auckland, New Zealand).

2.3. Noise exposure

Wistar rats (8–10 weeks old) were exposed to 8–12 kHz band-pass noise for 24 h at sound pressure levels that induce either temporary (100 dB SPL) or permanent (110 dB SPL) hearing loss. Noise exposures were carried out in a custom-built acoustic chamber (Shelburg Acoustics, Sydney, Australia) with internal speakers and external controls (sound generator and frequency selector). Sound intensity inside the chamber was tested using a calibrated Rion NL-40 sound level metre (Tokyo, Japan) to ensure minimal deviations of sound intensity. Control animals were housed in the animal facility at ambient sound conditions (45–55 dB SPL, 0.5–20 kHz). Cochlear tissues were collected for gene expression and immunohistochemical studies 24 h after noise exposure.

Table 1
PCR primers for screening of NADPH oxidase transcripts in the rat cochlea.

Target	GenBank accession No.	Forward primer	Reverse primer	Product length
Nox1	NM_053683	5′- ttccctggaacaagagatgg-3′ Position: 1483–1502	5′-gcaagtgtcaaccagcaaga-3′ Position: 2071–2052	589 bp
Nox2/Gp91^{phox}	NM_023965	5′- ccatatccgcattgttggcg-3′ Position: 1059–1078	5′- gtgttggggtgttgacttgc-3′ Position: 1589–1570	531 bp
Nox3	NM_001004216	5′- ccctgtggtcttgtatgcgt-3′ Position: 819–838	5′- tcccggcagatccagtagaa-3′ Position: 1334–1315	516 bp
Nox4	NM_053524	5′-tgtctgcttgtttggctgtc-3′ Position: 101–120	5′-ttctgggatcctcattctgg-3′ Position: 506–487	406 bp
Nox5/EF-hand calcium binding domain 5	XM_001080780	5′-aacagcagcgtgaggaagtt-3′ Position: 1661–1680	5′-tctctttccatcccctcctt-3′ Position: 2294–2275	634 bp
Duox1	NM_153739	5′-gcctgtcagctcatcaacaa- 3′ Position: 4622–4641	5′-gcactgctccaggaggttag-3′ Position: 5187–5168	566 bp
Duox2	NM_024141	5′-tggatggaaatggctttctc-3′ Position: 2606–2625	5′-gcgaggcaaagccatagtag-3′ Position: 3109–3090	504 bp