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siRNA-mediated knock-down of NOX3: therapy for hearing loss?

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Abstract

Cisplatin is a widely used chemotherapeutic agent that causes significant hearing loss. Previous studies have shown that cisplatin exposure is associated with increase in reactive oxygen species (ROS) in the cochlea. The inner ear expresses a unique isoform of NADPH oxidase, NOX3. This enzyme may be the primary source of ROS generation in the cochlea. The knockdown of NOX3 by pretreatment with siRNA prevented cisplatin ototoxicity, as demonstrated by preservation of hearing thresholds and inner ear sensory cells. *Trans*-tympanic NOX3 siRNA reduced the expression of NOX3 and biomarkers of cochlear damage, including transient receptor vanilloid 1 (TRPV1) channel and kidney injury molecule-1 (KIM-1) in cochlear tissues. In addition, siRNA against NOX3 reduced apoptosis as demonstrated by TUNEL staining, and prevented the increased expression of Bax and abrogated the decrease in Bcl2 expression following cisplatin administration. *Trans*-tympanic administration of siRNA directed against NOX3 may provide a useful method of attenuating cisplatin ototoxicity. In this paper, we review recent publications dealing with the role of NOX3 in ototoxicity and the effects of siRNA against cisplatin-induced hearing loss.

Keywords

Cisplatin; Cochlea; Hearing loss; NOX3; SiRNA

Introduction

Cisplatin ototoxicity

Cisplatin is an anti-neoplastic drug used to treat a variety of solid tumors including testicular, ovarian, cervical, bladder, lung, and head and neck cancers. Serious side effects may limit dosing of cisplatin. These include nephrotoxicity, neurotoxicity, and ototoxicity. Saline hydration and mannitol diuresis may help to prevent nephrotoxicity. However, no clinically proven treatments to prevent or ameliorate ototoxicity have been demonstrated. Some studies have reported hearing loss in as many as 75–100 % of patients treated with cisplatin [1]. The hearing loss from cisplatin is typically bilateral and permanent. It is particularly severe in young children less than 1 year old [2], especially those with neuroblastoma [3]. Other risk factors include the cumulative dose of cisplatin, nutritional status of the patient, noise exposure [4, 5], and radiation to the brain or skull base [6]. Hearing loss early in life can delay the development of speech as well as the social and cognitive development of children. It is crucial that treatments to prevent cisplatin ototoxicity be developed [3].

Cisplatin-induced hearing loss is sensorineural and affects the higher frequencies first. The primary targets for cisplatin in the cochlea are the outer hair cells (OHCs) of the organ of Corti [7, 8]. Damage can also include loss of cells in the spiral ganglion cells and cells of the lateral wall of the cochlea, particularly cells in the stria vascularis [9, 10].

Mechanisms of cisplatin ototoxicity

In vitro and in vivo studies of cisplatin ototoxicity demonstrated that cisplatin interacts with target tissues in the cochlea to produce reactive oxygen species (ROS) [11–15]. This may lead to depletion of antioxidant defenses which can no longer scavenge and neutralize ROS, such as superoxide [16]. Cisplatin can then accumulate in the cochlea and react with DNA, disrupting the synthesis of key proteins and leading to cell injury and apoptosis with resulting hearing loss. Antioxidant defense mechanisms in the cochlea include: superoxide dismutase, catalase, glutathione, glutathione peroxidase, and glutathione reductase [16]. The excess superoxide produced in cochlear tissues by cisplatin can react with nitric oxide to generate peroxynitrite which nitrosylates and inactivates proteins [17, 18]. Superoxide anion can also lead to the formation of hydroxyl radicals, which can react with polyunsaturated fatty acids in the cell membrane to generate the highly toxic aldehyde 4-hydroxynonenal (4-HNE). The latter can lead to increased calcium influx into OHCs, promoting apoptosis [19]. Superoxide can inactivate antioxidant enzymes [20] and can also cause cytosolic migration of Bax, leading to cytochrome c release from mitochondria and activation of caspases 3 and 9. Caspase-activated deoxyribonuclease is then activated, leading to DNA fragmentation [21]. A major source of ROS in the cochlea is the NADPH oxidase isoform Nox3 [15].

NOX3 in the inner ear

In the mouse, NOX3 is expressed in the inner ear and is essential for vestibular development and function. Paffenholz et al. [22] reported that mutations of the *het* locus affect the gene for Nox 3. These so-called head tilt mice (*het*) have impaired otoconia formation in the

utricle and saccule. These animals exhibit balance defects with an inability to detect linear acceleration or gravity resulting in abnormal behavior. They have oblique posture of the head, demonstrated as an abnormally “tilted” position of the head and behavioral manifestations of hyperactivity and circling. The otoconia defects result in abnormal performance in several tests of motor coordination, impaired air and ground righting reflexes, poor performance on the rotating rod and stationary balance beam tests, and an inability to swim [23]. These mutant mice were found to have normal hearing comparable to wild-type mice by acoustic startle audiometry [22].

In the cochlea, the organ of hearing, NOX3 has also been detected. An EST clone derived from the inner ear was used to carry out a detailed analysis of NOX3 distribution by real-time PCR and in situ hybridization [15]. NOX3 expression was very high in the organ of Corti and the spiral ganglion [15]. NOX3 mRNA was localized in the spiral ganglion by in situ hybridization [15]. HEK293 cells transfected with NOX3, NOXO1, and NOXA1 produced superoxide spontaneously and generated a dramatic increase in superoxide production following exposure to cisplatin. The production of superoxide by cisplatin was abolished by exposing these cells to diphenylene iodonium (DPI), an NADPH oxidase inhibitor [15]. It was concluded that, because of its tissue localization and dose response of the cisplatin effect on superoxide production in NOX3 transfected cells, that NOX3 is a likely source of ROS production in the cisplatin-treated cochlea [15].

Thus, rather than having a physiological role in hearing, NOX3 could play a pathological role in generating reactive oxygen species (ROS) that can react with target molecules within the cochlea, leading to irreversible damage to cells within the cochlea. ROS have been implicated in noise-, age-, and drug-induced cochlear injury, resulting in hearing loss [24–27].

Previous studies in experimental animals have shown that antioxidants such as *N*-acetylcysteine sodium thiosulfate, sodium salicylate, or lipoic acid provide some protection against cisplatin ototoxicity [28]. However, systemic administration of these drugs may compromise the therapeutic effect of cisplatin [29]. Therefore, recent experiments carried out in our laboratory have utilized *trans*-tympanic delivery of siRNA against NOX3. This would obviate the possibility of interference with the anti-tumor effect of cisplatin while delivering the protective agent to the site where it is needed to prevent ototoxicity.

Rats administered cisplatin (13 mg/kg by intraperitoneal injection) markedly up-regulated NOX3, measured in whole cochlear extracts by RT-PCR with specific primers for the rat [30]. NOX3 mRNA rose rapidly to nearly 2.5-fold by 24 h and remained elevated for at least 72 h following treatment. A marker of oxidative stress in the cochlea, Kidney Injury Molecule-1 (KIM-1) was up-regulated in the rat cochlea following cisplatin administration. KIM-1 mRNA expression was shown to be up-regulated in subregions of the rat cochlea after cisplatin treatment—in the organ of Corti, the lateral cochlear wall tissues, and the spiral ganglion cells. Furthermore, KIM-1 immunoreactivity was increased in outer hair cells. In vitro, increases in KIM-1 mRNA were found in a human kidney cell line (HK-2 cells) and in UB/OC1 cells, which are immortalized organ of Corti cells from mouse. These increases were prevented by DPI, an inhibitor of NADPH oxidase. These findings suggest

that KIM-1 activity is related to the activation of NADPH oxidase, presumably the NOX3 isoform [30].

Kim-1 is a type I cell membrane glycoprotein that is conserved in mammals including rodents, dogs, primates, and humans. *Kim-1* mRNA levels are elevated more than any other known biomarker gene after kidney injury. Urinary Kim-1 has been shown to be an early and sensitive biomarker for renal injury in a variety of acute and chronic rodent kidney injury models, resulting from drugs or ischemia [31].

NOX3 mRNA has been successfully knocked down in the cochlea of the rat following delivery to the middle ear by *trans*-tympanic injection of rodent NOX3 siRNA. Administration of this siRNA into the middle ear allows it to diffuse into the inner ear, presumably through the round window membrane. A dose-dependent knockdown of NOX3 mRNA was achieved without adverse effects on the rat hearing or inner ear morphology [32].

After cisplatin was administered to rats, pretreated with scrambled RNA, a nearly sevenfold up-regulation of NOX3 mRNA was observed. When NOX3 siRNA was administered 48 h prior to cisplatin injection (11 mg/kg ip), the up-regulation of NOX3 mRNA observed in the cochlea of rats was abrogated in a dose-dependent manner. The 0.3- μ g dose of siRNA was not effective, but the larger doses reduced the expression of NOX3 mRNA to control values [32].

The effects of siRNA against NOX3 in cisplatin-treated rats was evaluated using immunohistochemistry. Confocal images of mid-modiolar sections of rat cochlea were evaluated. There was a sevenfold increase in NOX3 immunofluorescence in cisplatin-treated rats pretreated with scrambled siRNA in OHCs compared to controls treated with scrambled RNA alone. Pretreatment with siRNA against NOX3 by transtympanic injection prior to cisplatin resulted in a dose-dependent decrease in the immunofluorescence of NOX3 in OHCs, spiral ganglion and stria vascularis [32].

TRPV1 and KIM-1 have been demonstrated to be stress markers for cisplatin ototoxicity [30, 33]. Cisplatin treatment increased the immunoreactivity for TRPV1 and KIM-1 immunoreactivity. TRPV1 and KIM-1 immunohistochemical staining showed co-localization of these markers in OHCs, SV, and SG cells. This increased expression of these two proteins was abolished by pretreatment with siRNA against NOX3 [2].

Cisplatin administration resulted in apoptosis in the cochlea as demonstrated by TUNEL staining in the OHCs, SV, and SG regions. Pretreatment with siRNA against NOX3 reduced the intensity of TUNEL staining in all three regions of the rat cochlea. No significant TUNEL positive staining was noted in rats treated with NOX3 siRNA alone [32]. Rats treated with cisplatin demonstrated a tenfold increase in the expression of Bax in the cochlea. Pretreatment with NOX3 siRNA reduced Bax up-regulation from cisplatin in a dose-dependent manner. Administration of siRNA against NOX3 alone had no effect on Bax expression. On the other hand, Bcl2 expression was markedly diminished in the cochlea of rats treated with cisplatin. This effect was reversed by pretreatment with transtympanic

siRNA for NOX3. The pro-apoptotic ratio of Bax to Bcl2 following cisplatin treatment was completely reversed in the cochlea of rats pretreated with siRNA [32].

Rats treated with cisplatin had a significant threshold shift in auditory brainstem response (ABR). This hearing loss was prevented by pretreatment with transtympanic siRNA against NOX3 in a dose-dependent manner (Fig. 1a). Morphological findings were consistent with the functional data. Scanning electron microscopy revealed significant damage to OHCs in the basal turn of the cochlea in cisplatin-treated rats. OHCs from rats pretreated with siRNA against NOX3 were found to have a dose-dependent preservation of OHC morphology [32] (Fig. 1b, c).

These observations demonstrate a key role for NOX3 in mediating cisplatin ototoxicity. The generation of ROS has been related to ototoxicity and nephrotoxicity from cisplatin. Cisplatin treatment results in up-regulation of NOX3 in the cochlea. The increased activity of this enzyme would result in increased superoxide radicals in the cochlea, leading to lipid peroxidation, damage to DNA and proteins, and hair cell death. The use of siRNA to knockdown NOX3 expression resulted in significant protection of the rat cochlea against cisplatin-mediated oxidative stress, hair cell damage, and hearing loss.

Paraquat ototoxicity

Paraquat (PQ) is a toxic herbicide known for its toxicity in various tissues, including lung [34] and the substantia nigra. The latter neurotoxic effect may result in Parkinson's disease [35]. Paraquat increased the expression of Nox1 in a concentration-dependent manner in rat dopaminergic N27 cells, which was prevented by apocynin or siRNA directed against NOX1 [5]. Intracellular NADPH donates an electron to PQ, catalyzed by NADPH oxidase. The PQ cation that is formed then is able to donate an electron to molecular oxygen, generating superoxide anion [34, 36].

The effects of PQ on the cochlea were evaluated in vitro and in vivo. Mouse cochlear organotypic cultures were treated with PQ for 24 h. M40403, a highly specific, nonpeptidyl mimetic of superoxide dismutase, was added to some cultures to inactivate the superoxide radical generated by PQ. The number of outer hair cells (OHC) and inner hair cells (IHC) decreased with increasing concentration of PQ (0.01–10 mM). M40403 (10 μ M) significantly increased OHC and IHC survival in cultures treated with 0.01–1.0 mM of PQ. This suggests that excess production of superoxide radical generated by PQ produced hair cell loss [37].

These findings were followed up in the chinchilla. Ten microliters of one of three concentrations of PQ was applied to the round window membrane of the chinchilla and compared to PBS. PQ-induced threshold shift was determined by measuring inferior colliculus-evoked potentials. Animals were tested at various times after PQ administration varying from 1 to 22 days. Pretreatment thresholds were deducted from post-treatment threshold determinations to obtain threshold shifts. The threshold shift at 22 days was used to determine permanent hearing loss. Dose response evaluation demonstrated that 10 mM PQ application resulted in the greatest PTS with mean shifts of 30–45 dB. Histologic

analysis of the cochlea revealed a dose-dependent loss of hair cells. The highest concentration of PQ caused the most loss of outer and inner hair cells, with the greatest loss at the base of the cochlea. The 5 mM PQ solution produced significantly fewer OHC loss and sporadic loss of IHC. The most dilute solution, 3 mM PQ, did not cause greater loss of hair cells than controls. The authors concluded that the cochlear damage and threshold shift induced by PQ was mediated by NADPH oxidase in the hair cells, and that superoxide generation by that NADPH oxidase is a potent mechanism for induction of hair cell death and hearing loss [36]. Since NOX3 is the predominant isoform of NADPH oxidase in the cochlea, it is likely that NOX3 was up-regulated by PQ, resulting in ototoxic injury.

Capsaicin ototoxicity

It has been demonstrated that cisplatin administration in rats caused an up-regulation of TRPV1, associated with hearing loss and cochlear damage [33]. The effects of capsaicin, the classical agonist of the TRPV1 channel, were studied in UB/OC1 cells. Capsaicin exposure resulted in ROS generation and stimulated release of intracellular Ca^{2+} release. This was associated with increased inflammation, shown by up-regulation of inducible nitric oxide synthase, tumor necrosis factor- α , and cyclooxygenase 2. The induction of these genes was maximal at 24 h but was transient. By 72 h, the expression of these genes was reduced to basal levels. Induction of these pro-inflammatory genes was associated with the induction of the signal transducer and activator of transcription (STAT-1). In turn, STAT-1 activation was dependent on ROS generated by NOX3. Knockdown of NOX3 in UB/OC1 cells attenuated the activation of STAT-1 by capsaicin. These observations were confirmed in rats that were treated with *trans*-tympanic injections of capsaicin. Capsaicin administration resulted in transient hearing loss, which was observed at 24 h and had recovered 72 h later. No damage to OHCs, SG, or SV was observed. *Trans*-tympanic administration of siRNA for NOX3 in rats produced a significant knockdown of mRNA for NOX3 in the cochlea. This treatment prevented the transient hearing loss and abrogation of STAT-1 activation, and the subsequent up-regulation of pro-inflammatory genes was attenuated [38].

Conclusions

NOX3 appears to play a critical role in mediating the production of damage to the cochlea that occurs with cisplatin administration. Knockdown of this gene by siRNA administration to the middle ear protects the cochlea against sensory cell damage, cell death, and hearing loss that may be caused by ROS generated by NOX3 up-regulation. This approach may open the way for novel treatments to prevent hearing loss caused by cisplatin. Other insults to the inner ear by noise, aminoglycoside antibiotics, aging, and environment toxins may damage the cochlea by activating NOX3, since they have been associated with increased ROS production (Fig. 2). Preliminary data (unpublished) from our laboratory demonstrate up-regulation of NOX3 following aminoglycoside antibiotic or noise exposure. There are no data relative to NOX3 changes in the cochlea associated with aging. Thus, blocking activation of NOX3 may prevent hearing loss from a variety of etiologies for hearing loss.

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Fig. 1.

Trans-tympanic injection of siRNA against NOX3 abrogates cisplatin-induced hearing loss in rat. **a** Pretreatment Auditory Brainstem Responses (ABRs) were recorded in naïve rats which were then administered scrambled siRNA (scramble) or siRNA against NOX3 (siNOX3). Cisplatin (11 mg/kg, intraperitoneally) was injected 48 h after siRNA administration. Post-treatment ABR thresholds were recorded 3 and 5 days after cisplatin administration and compared to pretreatment values. ABR threshold measured 3 days after scramble plus cisplatin showed a 35.5 ± 5 dB elevation which was reduced in a dose-dependent manner by siRNA against NOX3. **b** Scanning electron microscopy (SEM) of the basal turn of the cochlea showed protection of OHCs from cisplatin-induced damage by siRNA against NOX3 (siNOX3). *Trans*-tympanic injection of 0.6 μg of siRNA against NOX3 48 h prior to cisplatin administration protected OHCs from cisplatin-induced damage. SiNOX3 alone (0.6 μg) did not damage OHCs. *Arrows* indicate damaged OHCs. **c** Assessment of damage to OHCs by cisplatin from SEM images. Cisplatin damaged about 50 % of OHCs (scramble + cisplatin) group which was abrogated by *trans*-tympanic administration of 0.6 μg of siRNA against NOX3 (siNOX3). All experiments were repeated at least eight times. *Asterisk* (*) denotes statistically significant difference from the siNOX3 (0.6 μg) group, while (**) indicates significant differences between the scramble + cisplatin and siNOX3 (0.6 μg) + cisplatin treatment groups. Statistical significance was determined using the Student's *t* test and assessed at the $p < 0.05$ level [32]. (Republished with permission from Mary Ann Liebert, New Rochelle, NY 10801–5215, USA)

Fig. 2.

Flow chart showing various potentially harmful drugs, agents, or processes that could activate NOX3 in the cochlea, leading to excess ROS production, damage to cochlear tissues, resulting in cell death and hearing loss. It may be possible to protect the cochlea against some of these damaging influences by inhibition of NOX3, such as by administration of siRNA against NOX3