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ORIGINAL RESEARCH COMMUNICATION

Transtympanic Administration of Short Interfering (si)RNA for the NOX3 Isoform of NADPH Oxidase Protects Against Cisplatin-Induced Hearing Loss in the Rat

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Abstract

Cisplatin produces hearing loss in cancer patients. Reactive oxygen species (ROS) in the cochlea leads to lipid peroxidation, death of outer hair cells (OHCs), and hearing loss. The cochlea expresses a unique isoform of NADPH oxidase, NOX3, which serves as the primary source of ROS generation in the cochlea. Inhibition of NOX3 could offer a unique protective target against cisplatin ototoxicity. Here, we document that knockdown of NOX3 using short interfering (si) RNA abrogated cisplatin ototoxicity, as evidenced by protection of OHCs from damage and reduced threshold shifts in auditory brainstem responses (ABRs). Transtympanic NOX3 siRNA reduced the expression of NOX3 in OHCs, spiral ganglion (SG) cells, and stria vascularis (SV) in the rat. NOX3 siRNA also reduced the expression of transient receptor potential vanilloid 1 (TRPV1) channel and kidney injury molecule-1 (KIM-1), biomarkers of cochlear damage. Also, transtympanic NOX3 siRNA reduced the expression of *Bax*, abolished the decrease in expression of *Bcl2*, and reduced apoptosis induced by cisplatin in the cochlea. These data suggest that NOX3 regulates stress-related genes in the cochlea, such as TRPV1 and KIM-1, and initiates apoptosis in the cochlea. This appears to be the first study of the efficacy of transtympanic delivery of siRNA attenuating cisplatin ototoxicity. *Antioxid. Redox Signal.* 13, 589–598.

Introduction

Platinum drugs, such as cisplatin, are first-line agents for treatment of malignant solid tumors. However, the use of these agents is tempered by dose-limiting side effects such as ototoxicity, nephrotoxicity, and neurotoxicity. While nephrotoxicity may be alleviated by hydration, there are currently no known [except the study of a small select group of patients by (7)] therapeutic interventions for oto- and neurotoxicities. Cisplatin produces some degree of hearing loss, evidenced by elevations in hearing threshold, in 75%–100% of patients (20). The hearing loss is usually bilateral and irreversible and is dependent on the cumulative dose of the drug, the age of the patient, history of noise exposure (3), cranial irradiation (11), and nutritional status of the patient (16). The pediatric population appears more susceptible to hearing loss due to cisplatin (19).

Cisplatin-induced hearing loss is sensorineural, with loss of high frequency hearing occurring first. The outer hair cells (OHCs) of the organ of Corti represent the primary site for mediating hearing loss (27). This drug damages the OHCs (17) but damage can also include spiral ganglion (SG) neurons (6, 25, 32, 33) and cells of the stria vascularis (SV) (30). Cisplatin ototoxicity is linked to its ability to increase generation of reactive oxygen species (ROS). Studies with experimental animals have shown that various antioxidants (29), such as D-methionine (4, 18), lipoic acid (26, 28), amifostine (7, 12), and N-acetyl cysteine (5, 36) confer protection against cisplatin-induced hearing loss. However, systemic delivery of these agents could compromise the efficacy of cisplatin. Thus, there is a clear need for the development of a minimally invasive route of delivery of protective agents to the cochlea to reduce any potential side effects. Recent studies have identified the NOX3 isoform of NADPH oxidase as the main source of ROS generation utilized by cisplatin in the cochlea (2, 22). NOX3 appears to manifest some of its cytotoxicity through activation and induction of transient receptor potential vanilloid 1 (TRPV1) channel. Accordingly, round window administration of short interfering (si) RNA against TRPV1 reduces cisplatin-mediated ROS generation and ototoxicity (21). This latter finding suggests the existence of a feedback regulation of NOX3 activity by TRPV1.

In the present study, we employed localized delivery of NOX3 siRNA to the cochlea by transtympanic injections for reducing

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cisplatin ototoxicity. Our data indicate that NOX3 is an important and relevant target for knockdown in the treatment of cisplatin ototoxicity. Transtympanic administration of NOX3 siRNA produced significant and prolonged knockdown of NOX3, protected the OHCs and SG neurons from cisplatin-induced damage and attenuated hearing loss. Thus, targeting NOX3 via transtympanic delivery of siRNA could represent a novel approach in the treatment of cisplatin-induced hearing loss.

Methods

Reagents

The various reagents: cisplatin, and TRI reagent were purchased from Sigma-Aldrich (St. Louis, MO). The various antibodies were: NOX3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), TRPV1 antibody (Neuromics, Edina, MN), KIM-1 immune serum (22) and fluorescent tagged (dylight 488 and TRITC) secondary antibodies were purchased from Jackson Immuno Laboratories (West Grove, PA).

Transtympanic administration of siRNA

Male Wistar rats were anesthetized with ketamine/xylazine mixture. With the help of a Zeiss operating microscope, 28G–30G needles, 1/2–5/8 of an inch in length were used to make a single puncture of the tympanic membrane in the antero-inferior region, taking care not to touch the malleus or the facial nerve; 50 μl of solution were injected into the middle ear (siRNA was resuspended in 50 μl of sterile PBS to get the desired concentration). The rat was then left undisturbed for 15 min with the treated ear facing up. This procedure was then repeated in the other ear.

Animal procedures and sample collection

Male Wistar rats (250–300 g) were used for this study. Pretreatment auditory brainstem responses (ABRs) were performed immediately before transtympanic application of siRNA against NOX3 or a scrambled siRNA sequence which served as a control. Cisplatin (11 mg/kg) was administered 48 h after transtympanic siRNA administration, by intraperitoneal (i.p.) injection over a period of 30 min. The tympanic membrane was checked for perforation and it was observed that the puncture wound had healed completely by day 3 post cisplatin (or day 5 post siRNA injection in no treatment group) in all the rats. There was no evidence of middle ear effusion or infection in these animals. Post treatment ABR's were then performed 3 or 5 days following cisplatin administration. The cochleae were dissected and used for the extraction of total RNA, or perfused with 2.5% glutaraldehyde for morphological studies by SEM or with 4% paraformaldehyde for immunohistochemical analyses. For real-time PCR, tissues from six rats per group were assayed in duplicate. For scanning electron microscopy and immunohistochemistry, three cochleae per group were examined. For the ABR studies, at least eight animals per group were tested. All animal procedures were approved by the SIU Laboratory Animal Care and Use Committee.

Evoked potentials

Auditory brainstem responses were measured prior to administration and 72 h following cisplatin administration, as described previously (35). Animals were tested with a stimulus intensity series that was initiated at 0 dB SPL and reached a maximum at 90 dB SPL. The stimulus intensity levels were increased in 10 dB increments, and the evoked ABR waveforms were observed on a video monitor. The auditory stimuli included tone bursts at 8, 16, and 32 kHz with a 10 msec plateau and a 1 msec rise/fall time presented at a rate of 5/sec. Threshold was defined as the lowest intensity capable of evoking a reproducible, visually detectable response with two distinct waveforms and a minimum amplitude of 0.5 μV.

Morphological studies: scanning electron microscopy

Immediately after completion of post-treatment ABRs, deeply sedated rats were euthanized, their cochleae harvested and processed as described previously (14). Sputter coated cochleae were then viewed and photographed with a Hitachi S-500 scanning electron microscope (Hitachi Ltd., Tokyo, Japan).

Processing of cochleae for immunohistochemistry and intensity analysis

Cochleae were perfused with 4% paraformaldehyde, decalcified for 4–6 h by rapid decalcification using RapidCal Immuno, BBC Biochemical (Detroit, MI, http://www.bbcus.com), paraffin embedded, and sectioned. Samples were incubated with primary antibody (1:100 dilution) for 1 h at 37°C. Dylight488 or TRITC labeled secondary antibody (1:200 dilution) was used. For immunofluorescence imaging, slides were imaged and analyzed using an Olympus confocal microscope (Olympus America Inc., Melville, NY). Images captured were then analyzed using IMAGE J analysis software from NIH (http://rsbweb.nih.gov/ij/index.html). Statistical analyses were done using Students t test.

Hair cell count

Hair cell counts were performed using a modified version of the method described previously (18). Two representative areas of the basal turn and hook portion were photographed. In each area, OHCs were counted in an area that was ten pillar cell heads in length. The results are presented as the percent hair cell damage per cochlear turn.

Oligonucleotides

The rodent set of primers and siRNA were based on the homologous sequences in the rat and mouse cDNA sequences. The primers were purchased from Sigma Genosys (St.Louis, MO). Purified siRNA duplexes were purchased from Qiagen (Valencia, CA).

Rodent NOX3 (sense): 5′-GTGAACAAGGGAAGGCTCAT-3′
(antisense): 5′-GACCCACAGAAGAACACGC-3′,
Rodent-GAPDH (sense): 5′-ATGGTGAAGGTCGGTGTGAAC-3′
(antisense): 5′-TGTAGTTGAGGTCAATGAAGG-3′,
Rodent BAX (sense): 5′- ATGGCTGGGGAGACACCTGA-3′
(antisense): 5′- GCAAAGTAGAAGAGGGCAACC-3′
Rodent BCl2 (sense): 5′- CCTTCTTTGAGTTCGGTG-3′
(antisense): 5′- GAGACAGCCAGGAGAAAT-3′
Rat KIM1 (sense): 5′-TTCAAGTCTTCATTTCAGGCC-3′
(antisense): 5′- CTGCTCCGATAGGTGACTTGG-3′
Rodent TRPV1 (sense): 5′-CAAGGCTGTCTTCATCATCC-3′,
(antisense): 5′-AGTCCAGTTTACCTCGTCCA-3′,

Rodent NOX3 siRNA: Target sequence: 5′-AAGGTGGTG AGTCACCCATCT-3′. Custom siRNA was purchased from Quiagen. Scrambled siRNA was also procured from Human/Mouse starter kit (Qiagen).

RNA isolation

Cochleae were pared down to the bone, cracked open, and the soft tissue scooped out in 500 μl of TRI reagent. 0.1 ml of chloroform was added, and the tube was shaken vigorously for 15 seconds and centrifuged at 12,000 g for 15 min. RNA was extracted by washing the pellet with 0.5 ml ice-cold isopropanol followed by cold 75% diethylpyrocarbonate (DEPC) treated ethanol. The ethanol was removed and the tube was air dried briefly. The RNA pellet was resuspended in nuclease-free water and RNA levels were determined using optical density readings corresponding to wavelengths of 260, 280, and 320nm using a spectrophotometer (Eppendorf BioPhotometer, Hamburg, Germany).

Real time RT-PCR

One microgram of total RNA was converted to cDNA using an iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). The reaction mixture was set up as follows: 1 μg of total RNA, 4 μl of iScript reaction mix, 1 μl of iScript reverse transcriptase, nuclease-free water to bring the total volume to 20 μl. The reaction mix was incubated at 25°C for 5 min, 42°C for 30 min, and 85°C for 5 min,. This cDNA reaction mix was used for real time PCR.

PCR was set up as follows: 2 μl of cDNA, 0.5 μl of each primer (50 pM stock) and 12.5 μl of the iQ SYBR Green Supermix reagent (Biorad), adjusted to a total volume of 25 μl with DNAse/RNAse-free water. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used for normalization. Amplification and detection was performed with the Cepheid Smart Cycler Detection System (Sunnyvale, CA). Negative control reactions were set up as above without any template cDNA. On completion of amplification, melting curve analysis was performed by cooling the reaction to 60°C and then heating slowly 95°C, according to the instruction of manufacturer (Cepheid Smart Cycler). The cycle number at which the sample reaches the threshold fluorescent intensity was termed the cycle threshold (Ct). The relative change in mRNA levels between an untreated control (a) and a treated sample (b) was measured using the formula: $2^{(\text{Ct Target gene1-Ct GAPDH1})-(\text{Ct Target gene2-Ct GAPDH2})}$ (31). Negative controls for both the target genes and GAPDH were used for all reaction groups. Real time PCR products were analyzed on a 2% agarose gel to verify the correct product sizes and visualization of the amplified product was effected using the dye SyBr Green I (Invitrogen, Carlsbad, CA). Gene specific primer pairs were used for the various reactions and mRNA expression levels were normalized to the levels of GAPDH.

Apoptosis detection

For detecting apoptosis in the cochleae, Fluorimetric TdT-FragEL™ DNA fragmentation detection kit was used according to the manufacturer's instructions (EMD Biosciences, Gibbstown, NJ). Briefly, cochlear sections from different treatment groups were rehydrated according to the manufacturer's directions. Permeabilization with 20 μg/ml of proteinase K for 5 min, washed with 1× TBS and placed in 1× TdT equilibrating buffer for 20 min. Cochlear sections were then incubated in labeling reaction buffer containing Fluorescein-FragEL™ TdT labeling reaction mix and TdT enzyme for 60 min at 37°C in a humidified chamber. After one wash with 1× TBS mount with Vectasheild mounting media (Vector Laboratories, Burlingame, CA), fluorescence was detected using the Olympus confocal microscope.

Results

Transtympanic delivery of NOX3 siRNA reduced basal and cisplatin-induced NOX3 expression

In order to validate that transtympanic administration of siRNA produces knockdown in NOX3 RNA, different doses of siNOX3 (0.3, 0.6, and 0.9 μg) were administered to rats and NOX3 expression was evaluated by real time RT-PCR. The lowest dose of NOX3 siRNA used (0.3 μg) did not show any inhibition of NOX3 mRNA when compared to transtympanic administration of scrambled siRNA or untreated cochleae. However, administration of the higher doses of NOX3 siRNA (0.6 and 0.9 μg) reduced NOX3 expression to 0.3 ± 0.2 and 0.3 ± 0.1-fold, compared to control scrambled siRNA (Fig. 1).

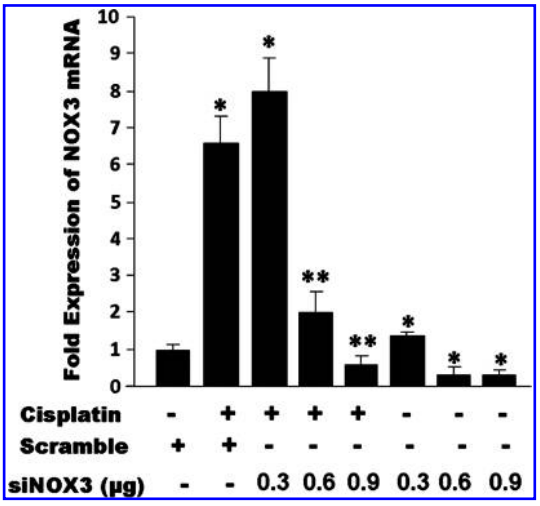

FIG. 1. Transtympanic administration of NOX3 siRNA against NOX3 inhibits cisplatin-induced NOX3 expression in the rat cochlea. Various doses of NOX3 siRNA (siNOX3; 0.3, 0.6, and 0.9 μg) were injected transtympanically into the cochleae of male Wistar rats, followed by cisplatin (11 mg/kg, i.p) 48 h later, and cochleae were collected 72 h post cisplatin treatment. Total RNA was extracted, converted to cDNA, and used for real time qRT-PCR for NOX3. GAPDH was used for normalization. Cisplatin increased NOX3 mRNA expression ~6.6-fold, which was abrogated by 0.6 and 0.9 μg of NOX3 siRNA, but not by 0.3 μg dose. NOX3 siRNA, added alone at both 0.6 and 0.9 μg doses reduced NOX3 mRNA to ~0.3-fold compared to no treatment as well as scrambled siRNA (scramble) alone groups. Data represented as mean ± SEM of at least six rat cochleae, each performed in duplicates. Statistical significance, assessed at $p < 0.05$ level, was measured using analysis of variance. Asterisk (*) shows significant difference from scrambled siRNA-treated cochleae, while (**) indicates significant difference in values between cisplatin treatment versus NOX3 siRNA + cisplatin treatments.

Knockdown of NOX3 protein was more difficult to discern from immunocytochemistry due to the low levels of proteins expressed under the basal condition (see Fig. 2A).

Assessment of cochleae 3 days after cisplatin administration indicated an 8.0 ± 0.9-fold increase in NOX3 mRNA. Therefore, to further validate knockdown of NOX3 mRNA, we assessed the cochlear expression of NOX3 mRNA following cisplatin administration. No knockdown of cisplatin-induced NOX3 expression was observed following pretreatment with siNOX3 (0.3 μg). However, pretreatment with 0.6 and 0.9 μg siNOX3 resulted in 2.0 ± 0.5 and 0.6 ± 0.2-fold changes in NOX3 expression, respectively, as compared to scrambled siRNA alone (Fig. 1).

To further validate the decrease in NOX3 protein by siNOX3 *in vivo*, fluorescent immunohistochemistry for NOX3 protein was performed on mid-modiolar sections of the cochleae 3 days after cisplatin administration. Images were visualized by confocal microscopy and fluorescent intensities of NOX3 were quantitated by the Image J analysis program. Immunoreactivity analysis indicated a 7-fold increase in NOX3 immunofluorescence in the cochleae of rats treated with cisplatin + scrambled siRNA in the OHCs compared to scrambled siRNA alone. Transtympanic injections of NOX3 siRNA 48 h prior to cisplatin treatment (11 mg/kg, i.p) significantly reduced cisplatin-induced NOX3 immunoreactivity in the OHCs in a dose dependent manner (Fig. 2A). NOX3 siRNA (0.3 μg, 0.6 μg, and 0.9 μg) reduced cisplatin-induced NOX3 immunoreactivity to 4, 0.6 and 0.8-fold, respectively (Fig. 2B). NOX3 siRNA (0.6 μg) administered alone also significantly reduced basal NOX3 immunoreactivity in OHCs, in accordance with knockdown of basal NOX3 expression in the OHCs. Transtympanic NOX3 siRNA also decreased NOX3 immunoreactivity in SV as well as in the SG cells at all the three doses tested.

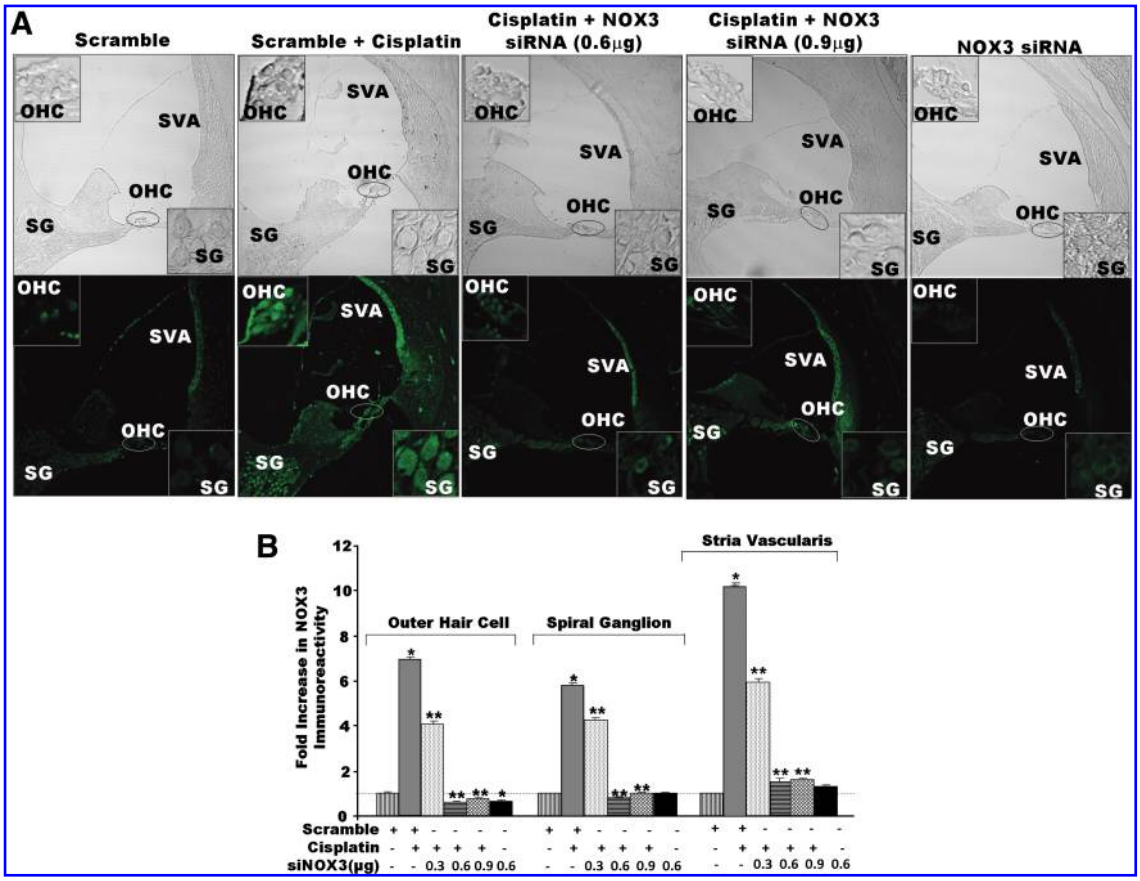

FIG. 2. NOX3 siRNA inhibits cisplatin-induced NOX3 immunoreactivity in the rat cochleae. Varying doses of NOX3 siRNA (0.3, 0.6, and 0.9 μg) or scrambled siRNA (scramble) were injected transtympanically into the cochlea of male Wistar rats, followed by cisplatin (11 mg/kg, i.p) 48 h later. Cochleae were collected 72 h following cisplatin treatment, perfused with 4% paraformaldehyde, decalcified, paraffin fixed, and mid-modiolar sections were obtained for immunohistochemistry. Sections were incubated with NOX3 primary antibody, followed by incubation with DyLight 488 fluorescent-tagged secondary antibody and visualized by confocal microscopy. (**A**) NOX3 immunoreactivity was observed in the OHCs, SV, and SG cells. Cisplatin-increased NOX3 immunoreactivity in these regions was decreased dose-dependently by NOX3 siRNA. (**B**) Fluorescent intensity analysis of the images in (**A**) showing increased fluorescence by cisplatin (~7 fold), compared to scramble and reductions by transtympanic injections of NOX3 siRNA (siNOX3). *Inset* shows 500-fold magnification of OHCs and SG cells. Abbreviations: OHCs, outer hair cells; SV, stria vascularis; SG, spiral ganglion. Asterisk (*) shows significant differences between control (scrambled siRNA treated) and treated cochleae, while (**) shows significant difference between cisplatin treatment versus siNOX3 + cisplatin. Statistical significance ($p < 0.05$) was determined by analysis of variance.

Transtympanic injections of NOX3 siRNA decreased other markers of ototoxicity in the rat cochlea

Previous data from our lab show that TRPV1 (22) and KIM-1 (21) serve as important markers of cisplatin ototoxicity. We therefore determined whether transtympanic administration of NOX3 siRNA could suppress these markers of ototoxicity.

Immunolabeling studies showed co-localization of TRPV1 and KIM-1 in the OHCs, SV and SG cells (Fig. 3A, shown as insets). Graphical representation of intensity analysis of both proteins indicated that cisplatin increased TRPV1 (green) KIM-1 (red) immunoreactivity by 3.4 and 4.2-fold, respectively. Pre-treatment with NOX3 siRNA (0.6 μg) abolished these increases in TRPV1 and KIM-1 immunoreactivity, the levels being 0.4 ± 0.1-fold and 0.6 ± 0.1-fold, respectively, compared to scramble + cisplatin treatment group (Fig. 3B,C).

Transtympanic administration of scrambled siRNA + cisplatin increased TRPV1 expression by 5.0 ± 0.5-fold and KIM-1 mRNA expression by 4.3 ± 0.2-fold compared to scrambled siRNA alone. As before, we observed 0.3 μg NOX3 siRNA did not alter cisplatin-induced TRPV1 expression (5.4 ± 0.4-fold increase) and KIM-1 (4.1 ± 0.4-fold increase), compared to the scrambled siRNA + cisplatin group. However, cisplatin-induced increases in TRPV1 and KIM-1 expression were lowered significantly by transtympanic administration of 0.6 μg siNOX3 (1.7 ± 0.4 and 1.2 ± 0.2-fold increases, respectively) and 0.9 μg (0.5 ± 0.1-fold and 0.6 ± 0.1-fold, respectively) (Fig. 3D). Transtympanic administration of increasing doses of NOX3 siRNA reduced basal expression of both TRPV1 and KIM-1 (0.3 μg: 1 ± 0.1 and 0.8 ± 0.1-fold, respectively; 0.6 μg: 0.6 ± 0.1 and 0.5 ± 0.1-fold, respectively; 0.9 μg: 0.4 ± 0.1 and 0.5 ± 0.1-fold, respectively).

Transtympanic injections of NOX3 siRNA inhibited cisplatin-induced apoptosis in the cochlea

To further examine the consequences of transtympanic administration of siNOX3, we determined its ability to reduce cisplatin-mediated apoptosis in the cochlea by terminal deoxynucleotidyl transferase mediated dUTP nick-end-labeling (TUNEL) assays. Basal fluorescence was observed in OHCs of animals administered scrambled siRNA by transtympanic injections. No significant TUNEL positive staining was observed in other regions of the cochlea, including the SG cells and SV. Increased TUNEL staining was observed in cochleae obtained from rats pretreated with scrambled siRNA, followed by cisplatin for 3 days (Fig. 4A). TUNEL positive staining was observed in the OHCs, SV, and SG cells. Pretreatment of cochlea with NOX3 siRNA (0.6 μg) reduced the intensity and distribution of TUNEL staining in these three regions. There were substantial reductions in staining in the SV, but moderate reductions in the OHCs and SG cells (Fig. 4A). However, no significant TUNEL positive cells were observed following cisplatin administration in the cochleae obtained from rats pretreated with 0.6 or 0.9 μg siNOX3, suggesting attenuation of the apoptotic cascade.

Real time PCR studies to quantitate genes associated with apoptosis indicate a 10 ± 0.8-fold increase in *Bax* expression, a pro-apoptotic gene, in the cochlea by cisplatin (Fig. 4B). Pretreatment of rats by transtympanic injections of NOX3 siRNA at doses of 0.3, 0.6, and 0.9 μg, respectively, reduced *Bax* expression to 4.6 ± 0.4, 0.6 ± 0.1, and 0.8 ± 0.3-fold. Transtympanic administration of these doses of NOX3 siRNA alone did not affect *Bax* expression, compared to treatment with scrambled siRNA alone. The expression of anti-apoptotic *Bcl2* gene was significantly reduced by cisplatin to 0.1-fold (levels in cochlea treated with scrambled siRNA being 1). Transtympanic NOX3 siRNA prior to cisplatin restored the expression of *Bcl2* to 1.2 ± 0.3, 1.5 ± 0.3, and 1.0 ± 0.3-fold at doses of 0.3, 0.6, and 0.9 μg, respectively. Taken together, these studies confirm that NOX3 siRNA at doses $\geq$0.6μg narrow the $\sim$100 fold difference in *Bax:Bcl2* mRNA expression induced by cisplatin to basal levels, thus restoring a healthy environment in the organ of Corti.

Transtympanic injections of NOX3 siRNA reduced cisplatin-induced hearing loss

The previous observations that TRPV1 siRNA applied to the round window of rats after surgical exposure reduces the expression of stress and apoptotic markers in the cochlea suggest that this agent would also protect against cisplatin ototoxicity (22). We therefore determined whether transtympanic application of NOX3 siRNA protected against cisplatin-induced hearing loss in rats by first examining changes in ABR thresholds. The hearing thresholds of male Wistar rats were first determined and animals were then administered either scrambled siRNA or NOX3 siRNA (at doses 0.3, 0.6, or 0.9 μg). Forty-eight hours later, rats were administered cisplatin (11 mg/kg, i.p) and post-treatment ABRs were determined 3 and 5 days later. We observed a 35 ± 5 dB ABR threshold shift produced by cisplatin on at post-treatment day 3 and a 49 ± 5 dB shift on post-treatment day 5 (Fig. 5A) in rats administered a scrambled siRNA sequence by transtympanic injections. In rats administered NOX3 siRNA by transtympanic injection, however, the responses to cisplatin were reduced in a dose-dependent manner. ABR threshold shifts produced by cisplatin were reduced to 23 ± 5 dB (for testing on both days 3 and 5) following administration of 0.3 μg NOX3 siRNA and were significantly reduced at the 0.6 and 0.9 μg doses in animals tested on both days 3 and 5.

Morphological examination of the cochlea by scanning electron microscopy (SEM) indicated a significant degree of damage to the OHCs at this dose of cisplatin as indicated by arrows (Fig. 5B) from the basal turn of the organ of Corti. This area generally shows increased susceptibility to oxidative damage produced by cisplatin. Transtympanic administration of 0.3 μg NOX3 siRNA was not effective in reducing damage to OHCs. However, transtympanic injections of 0.6 and 0.9 μg doses produced almost complete protection from cisplatin-mediated OHC damage (data not shown). This is supported by counting damaged OHCs. When cisplatin was administered with a scrambled siRNA sequence a $\sim$50% damage of hair cells were observed. While pretreatment with 0.3 μg siNOX3 did not affect the degree of hair cell damage, significant protection was observed with the 0.6 and 0.9 μg doses.

Discussion

This study demonstrates an essential role of NOX3 in mediating cisplatin ototoxicity and provides a good rationale for targeting this protein for knockdown to ameliorate drug-induced ototoxicity. The generation of ROS has been implicated in cisplatin ototoxicity and nephrotoxicity (29). Cisplatin administration has been previously associated with upregulation of NOX3 in the cochlea (2, 21). ROS could also

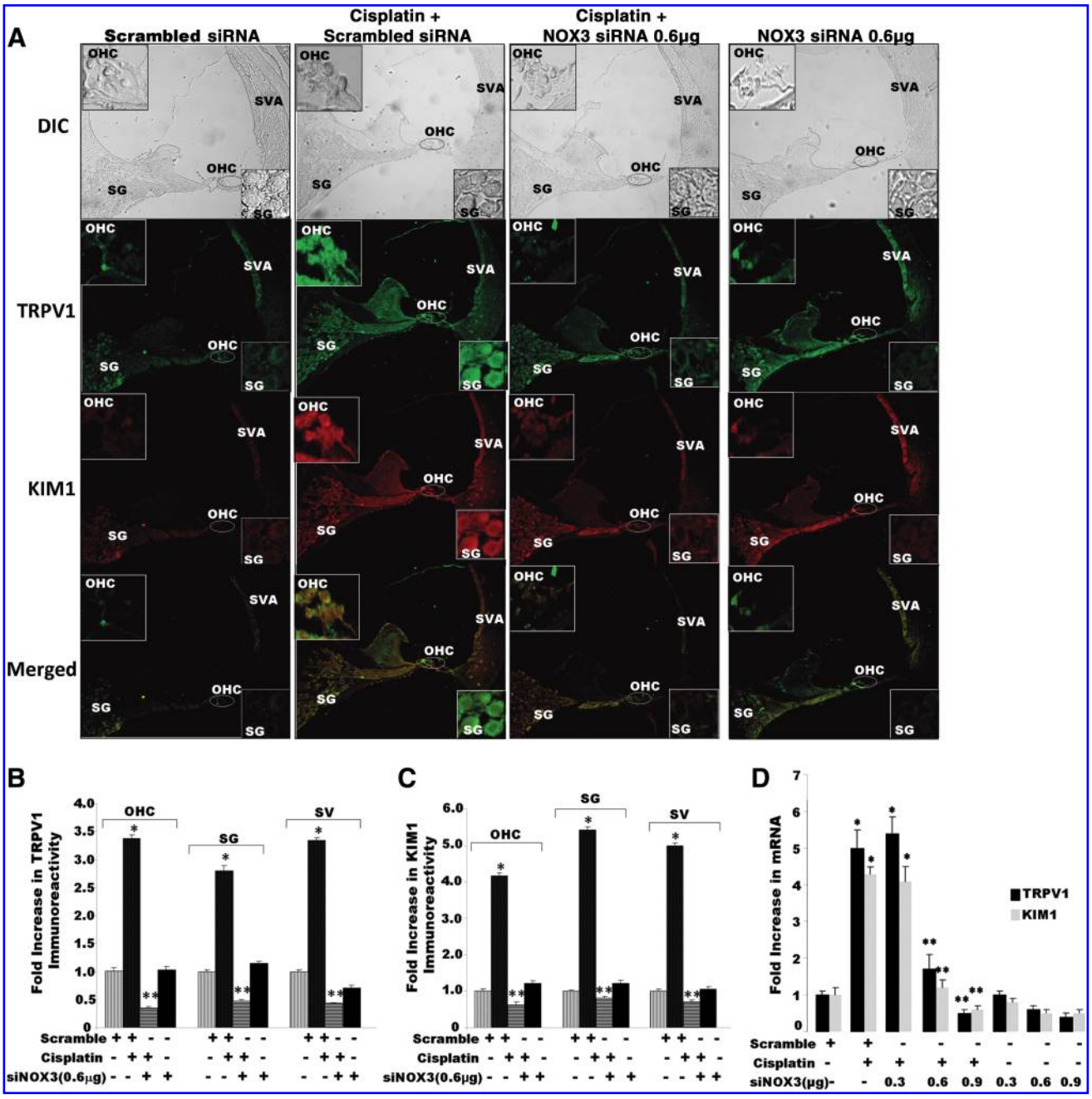

FIG. 3. Transtympanic NOX3 siRNA decreases markers of cisplatin ototoxicity. Rats were administered either scrambled siRNA (scramble) or NOX3 siRNA (siNOX3) by transtympanic injections, followed by cisplatin (11 mg/kg, i.p.) 48 h later. Cochleae were dissected out and flash-frozen for RNA analysis or perfused with 4% paraformaldehyde, decalcified, and sectioned for immunohistochemistry. (**A**) Composite cochlear images showing TRPV1 and KIM-1 immunolabeling. TRPV1 (*green*) and KIM-1 (*red*) fluorescence are presented individually and as merged images (*yellow*). *Upper panel* shows the differential interference contrast (DIC) images of the immunolabeled fields. The images are presented as a 100-fold magnification, while *insets* show 500-fold magnification of OHC and SG. Cisplatin increased TRPV1 and KIM-1 immunofluorescence in OHC, SG, and SV. Transtympanic administrations of siNOX3 (0.6 μg) inhibited the induction of both TRPV1 and KIM-1 by cisplatin. (**B**, **C**) Fluorescent intensity analysis for TRPV1 and KIM-1 immunolabeling, respectively, in the cochleae from (**A**). Transtympanic siNOX3 (0.6 μg) decreased cisplatin-induced TRPV1 and KIM-1 immunoreactivity in OHC. (**D**) Graphical representation of TRPV1 and KIM-1 mRNA expression by qRT-PCR. Cisplatin increased TRPV1 and KIM-1 expression which was reduced by siNOX3. Data are presented as the mean ± SEM of whole cochleae from three rats. Asterisk (*) shows significant differences as compared to scramble siRNA (scramble)-treated cochleae, while (**) shows significant difference in values between cisplatin treatment versus siNOX3 + cisplatin treatments ($p < 0.05$). Abbreviations: OHCs, outer hair cells; SV, stria vascularis; SG, spiral ganglion.

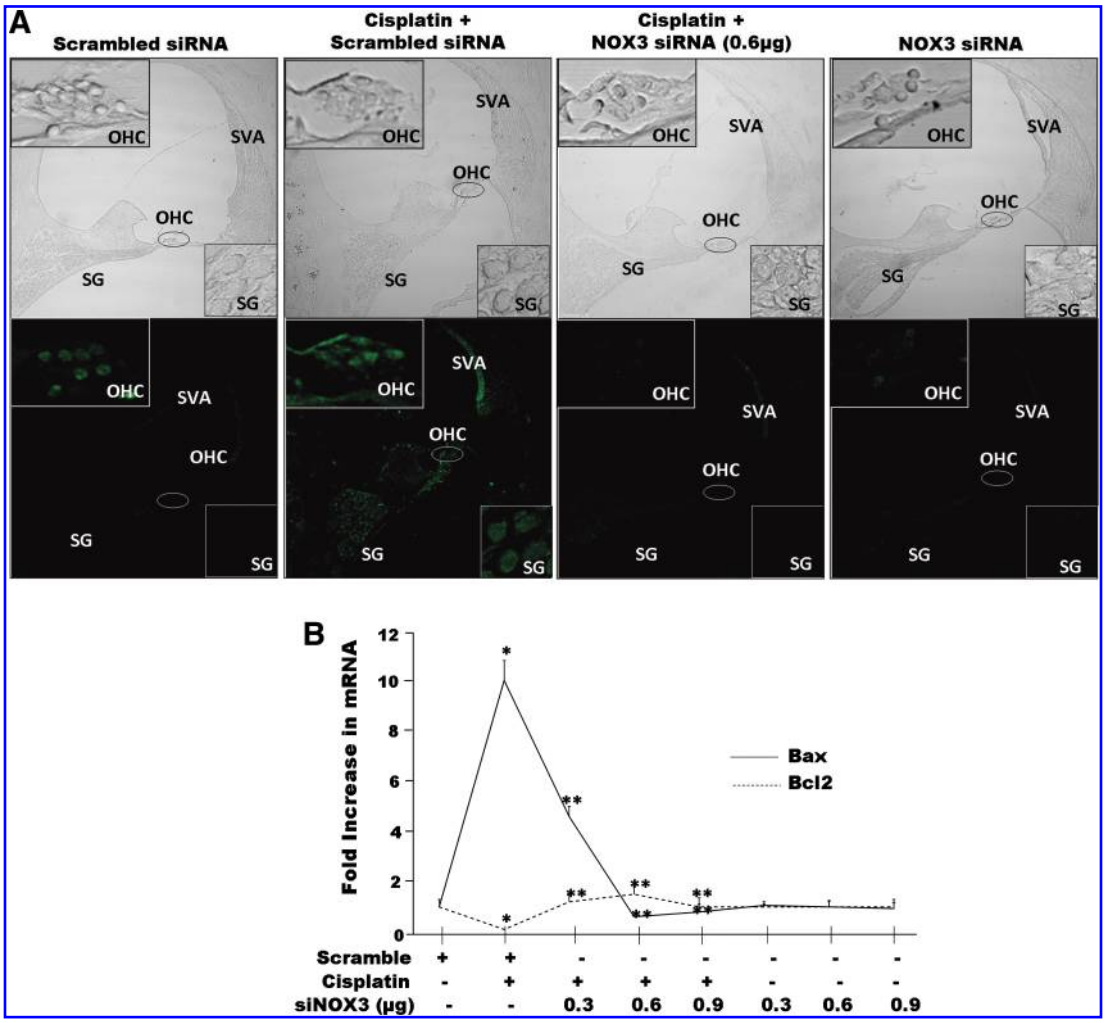

FIG. 4. Transtympanic administration of NOX3 siRNA decreased cisplatin-induced apoptosis in the cochlea. (**A**)TUNEL assay was performed on mid-modiolar sections of the cochlea from the various treatment groups and visualized by confocal microscopy. Fluorescent images of the cochlea are presented at 100-fold magnification, while insets were magnified 500-fold. (**B**) Expression of *Bax* (pro-apoptotic protein) and *Bcl2* (anti-apoptotic protein) was measured by qRT-PCR. *Bax* mRNA was increased by cisplatin, but this was dose-dependently reduced by pretreatment with NOX3 siRNA (siNOX3). *Bcl2* expression was decreased by cisplatin but recovered to baseline levels by pretreatment with different doses of siNOX3. Data are presented as the mean ± SEM of 3 rats per group. Asterisk (*) indicates significant differences between scramble siRNA (scramble) and cisplatin + scramble groups, while (**) indicates significant difference between cisplatin and siNOX3 + cisplatin treatment groups ($p < 0.05$).

function as a positive feedback regulator of NOX3 expression, accelerating the increases in ROS production. The NOX3 isoform of NADPH oxidase is the primary source of ROS generation in cochlear explants (2). We have reported that NOX3 is an essential component of cisplatin-mediated ROS generation in UB/OC1 cells and in the rat model (22) and that NOX3 is induced by cisplatin in an ROS-dependent manner. Thus, NOX3 could constitute the major source of ROS production and lipid peroxidation in the cochlea following cisplatin exposure. Thus, the selection of knockdown of NOX3 expression using siRNA was considered as a therapeutic strategy to prevent ROS generation, thereby attenuating cochlear damage following cisplatin injection.

Our data show that a single transtympanic injection of siRNA against NOX3 reduced cisplatin-induced ABR threshold shift in the rat in a dose-dependent manner. Scrambled siRNA administered prior to cisplatin had no protective effect, indicating a requirement for specific knockdown of NOX3. This protective effect was confirmed by morphological studies showing a significant reduction of damage to OHCs. SEM data showed nearly complete protection of OHCs by the higher doses of siRNA (0.6 and 0.9 μg) given prior to cisplatin administration. Molecular studies confirmed the dose-dependent knockdown of NOX3 mRNA expression in the cochlea by these higher doses of siRNA. The siRNA directed against NOX3 also reduced the upregulation of NOX3 immunoreactivity in a dose-dependent manner in the OHCs, SV and SG cells of rats treated with cisplatin.

Previous studies from this laboratory have shown that cisplatin induces the expression of KIM-1 in the rat cochlea (22). KIM-1 is a novel membrane protein in the renal proximal tubule epithelial cells that structurally resembles mucosal addressin cell adhesion molecule one (MAdCAM-1). KIM-1 is induced in response to insults to the kidney, including

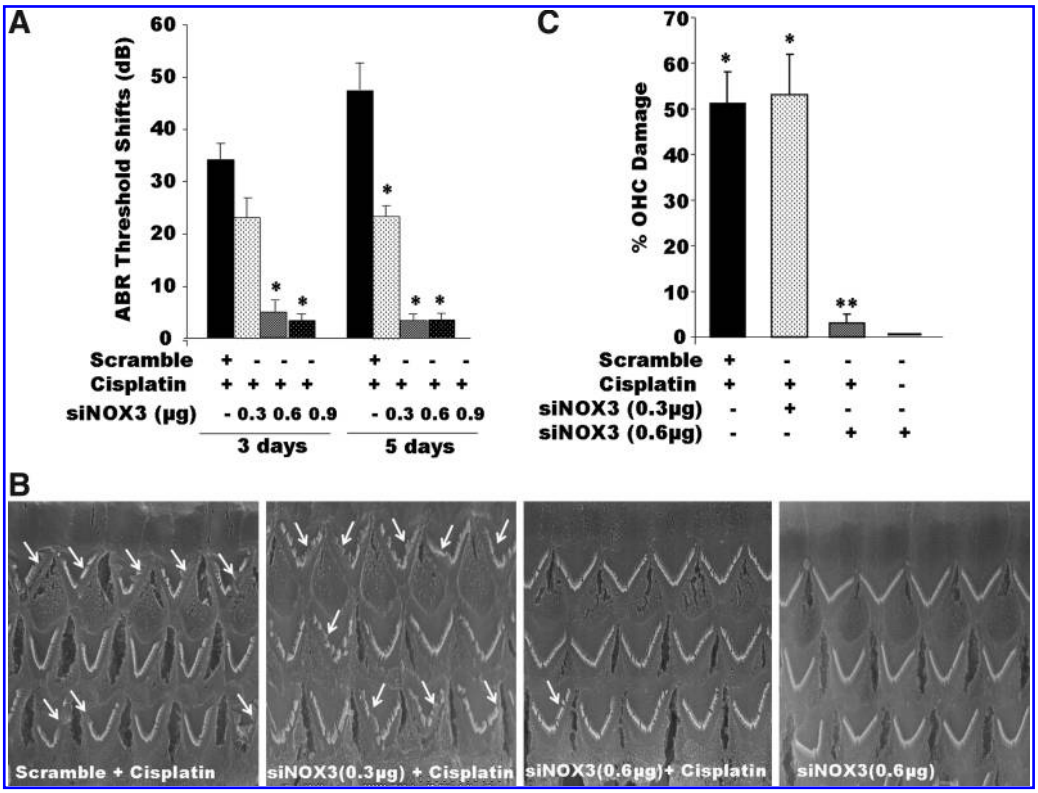

FIG. 5. Transtympanic injection of NOX3 siRNA abrogates cisplatin-induced hearing loss in the rat. (**A**) Pretreatment ABRs were recorded on naïve rats which were then administered scrambled siRNA (scramble) or NOX3 siRNA (siNOX3). Cisplatin (11 mg/kg, i.p) was injected 48 h following siRNA treatments. Post treatment ABR thresholds were recorded 3 and 5 days following cisplatin administration and compared to pretreatment values. ABR threshold measured 3 days post scramble + cisplatin administration showed a 35 ± 5 dB elevation which was reduced dose-dependently by siNOX3. The threshold shift obtained 5 days after scramble + cisplatin administration was 49 ± 5 dB, which was dose-dependently reduced by siNOX3. (**B**) SEM of the basal turn of cochlea showing protection of OHCs from cisplatin-induced damage by NOX3 siRNA (siNOX3). Transtympanic injection of 0.6 μg of siNOX3 48 h prior to cisplatin administration protected OHCs from cisplatin-induced damage. siNOX3 (0.6 μg) alone did not produce damage to OHCs. *Arrows* indicate damaged OHCs. (**C**) Assessment of damage to OHCs by cisplatin from SEM images. Cisplatin damaged $\sim$50% of OHCs (scramble + cisplatin) group which was abrogated with transtympanic administration of 0.6 μg NOX3 siRNA (siNOX3). All experiments were repeated at least eight times. Asterisk (*) denotes statistically significant difference from the siNOX3 (0.6 μg) group, while (**) shows significant differences between the scramble + cisplatin and siNOX3 (0.6 μg) + cisplatin treatment groups. Statistical significance was measured using the Students *t*-test and assessed at the $p < 0.05$ level.

ischemia-reperfusion and exposure to nephrotoxic agents, such as aminoglycoside antibiotics and cisplatin (13). This molecule appears to provide an anchor for the attachment of new renal epithelial cells to facilitate regeneration of kidney structure and function (1). The upregulation of KIM-1 in the cochlea appears to be triggered by an increase in ROS generation following cisplatin administration (22) and thus serves as an early indicator of oxidative stress. The present study provides additional support for this hypothesis. A dose-dependent attenuation of the upregulation of KIM-1 mRNA expression in the rat cochlea following cisplatin administration was observed upon NOX3 siRNA pretreatment. This finding was further confirmed by immunohistochemical localization of KIM-1 immunoreactivity in OHCs, SV, and SG cells of the rat cochlea. Attenuation of cisplatin-induced KIM-1 expression by NOX3 siRNA serves as an additional indicator of a reduction in oxidative stress in the cochlea.

We have also previously shown that cisplatin increases the expression of TRPV1 in the rat cochlea and that ROS can activate and increase the expression of TRPV1. siRNA against TRPV1 applied to the round window after surgical exposure decreased both KIM-1 and NOX3 expression both *in vitro* and *in vivo* (21). In the present study, we observed that siRNA against NOX3 abrogated the upregulation of TRPV1 mRNA by cisplatin. We further found that cisplatin-induced increase of TRPV1 immunoreactivity in the cochlea (OHCs, SV, and SG cells) was attenuated in a dose-dependent manner. Double-label immunohistochemical studies of the cochleae of cisplatin treated rats showed that the increased immunolabeling for both TRPV1 and KIM-1 occurred in the same cell layers of the cochlea (OHCs, SV, and SG cells). The increase in immunoreactivity for both proteins following cisplatin administration was abrogated by siRNA against NOX3. These data support the contention that NOX3 siRNA reduces the overall stress to the cochlea induced by cisplatin. Co-localization of KIM-1 and TRPV1 may indicate potential interaction between these two molecules under normal conditions and following oxidative stress and support the contention that ROS controls the expression of these proteins.

Cisplatin has been shown to cause apoptosis in cochlear cells *in vitro* and *in vivo*. In this study we show that cisplatin induced apoptosis in the OHCs and SG cells with the fluorescent TUNEL assay. The TUNEL staining was dramatically reduced in a dose-dependent manner when siRNA against NOX3 was administered. These findings were further supported by results showing that cisplatin induced the expression of the pro-apoptotic *Bax* gene while downregulating the anti-apoptotic *Bcl2* gene, causing a large increase (~100 fold) in the ratio of Bax to Bcl2 proteins. This strong pro-apoptotic effect was abrogated in a dose-dependent manner by the transtympanic administration of siRNA for NOX3. We propose that the coordinated activation and/or induction of NOX3 and TRPV1 could be major factors in mediating apoptosis in the cochlea and cisplatin ototoxicity.

The advantages of siRNA therapy include its ability to selectively knock down the expression of proteins and to maintain this knockdown for an extended period of time. The transtympanic route of administration of NOX3 siRNA allows for localization of its effect to the cochlea, thereby limiting systemic side effects. Our study provides evidence for protection against hearing loss and damage to the OHCs up to at least 5 days post treatment. This relatively long duration of protection could support the use of NOX3 siRNA when long-term protection is needed against drug-induced hearing loss. Since ROS has also been implicated in mediating noise-induced hearing loss (24), NOX3 siRNA could offer protection against this condition. NOX3 siRNA offers an additional benefit by reducing ROS generation within the cochlea, presumably without diminishing the chemotherapeutic action of cisplatin, a concern associated with the use of systemically administered antioxidant drugs to treat cisplatin ototoxicity. Since ROS controls a number of genes implicated in cisplatin ototoxicity, knockdown of NOX3 would directly contribute to reduced expression of these genes which could further contribute to otoprotection. As such, knockdown of NOX3 could afford protection by not only reducing ROS generation but also by reducing TRPV1 expression, a protein which is also dependent on ROS that contributes to cisplatin ototoxicity (21).

To our knowledge, this is the first publication demonstrating the efficacy of transtympanic delivery of naked siRNA to attenuate cisplatin ototoxicity. This relatively noninvasive route of administration could prove to be effective in protecting against cisplatin-induced hearing loss without compromising the therapeutic efficacy of this important anti-tumor agent. Clinically, the transtympanic route of drug administration is the most frequently used first-line treatment for vertigo associated with Meniere's disease with aminoglycosides (10, 23, 34), tinnitus (34), and idiopathic sudden sensorineural hearing loss with corticosteroids (8, 9, 15, 38), and autoimmune sensorineural hearing loss with infliximab, a monoclonal antibody against tumor necrosis factor alpha (37). This route for drug administration can be used in the outpatient clinic under local anesthesia. The transtympanic delivery of siRNA against NOX3 prior to cisplatin chemotherapy could ameliorate cisplatin ototoxicity in patients. The ease of administration of the siRNA via transtympanic injection would suggest that this could be a potentially useful therapeutic intervention to protect against cisplatin ototoxicity.

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Author Disclosure Statement

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Abbreviations Used

ABR = auditory brainstem responses
GAPDH = glyceraldehyde phosphate dehydrogenase
KIM-1 = kidney injury molecule-1
MAdCAM-1 = mucosal addressin cell adhesion molecule-1
OHC = outer hair cell
ROS = reactive oxygen species
SG = spiral ganglia
siRNA = short interfering RNA
SV = stria vascularis:
TRPV1 = transient receptor potential V1
TUNEL = terminal deoxynucleotidyl transferase mediated dUTP nick-end-labeling

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