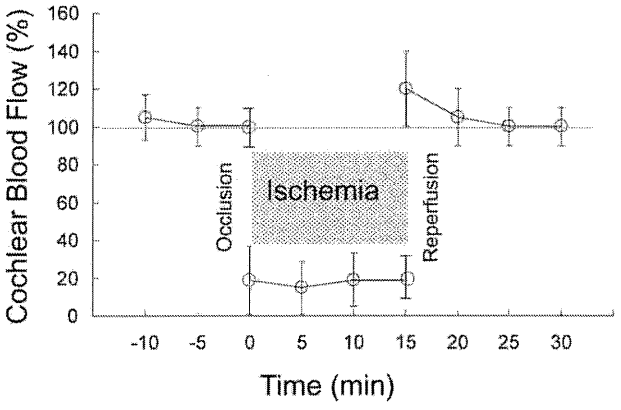

FIG. 3. Occlusion of the bilateral vertebral arteries severely reduced cochlear blood flow before reperfusion, which then increased over the preischemic level after reperfusion, and returned to baseline shortly thereafter.

treatment group. This means that hearing loss remained more severe in the order of animals treated with RBC or saline, l-LEH, and H-LEH at 32 kHz on day 7 (right). In contrast, hearing impairment severity was milder in each group with the h-LEH-treated animals recovering to the preischemic level at 8 kHz.

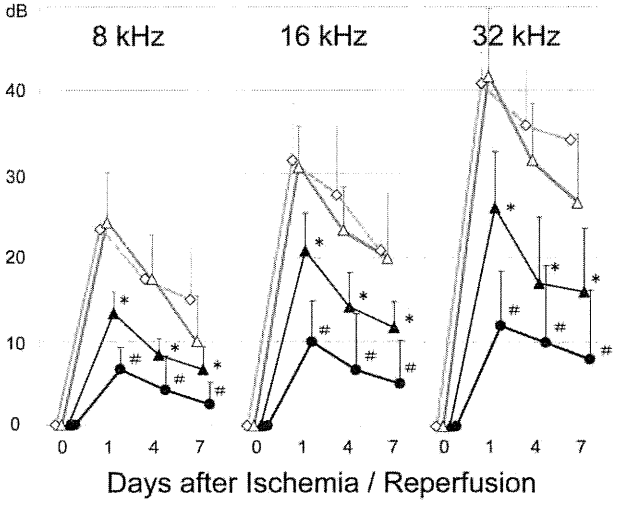

FIG. 4. The average increase in ABR threshold was significantly milder (all $P < 0.05$) in the order of animals receiving h-LEH (closed circles), l-LEH (closed triangles), and RBCs (open triangles) or saline (open rectangles). No difference was observed between the latter two groups. Among the frequencies tested, the higher the frequency, the more severe was the magnitude of hearing loss and the difference among the treatment groups. Hearing loss was most prominent on day 1, then decreasing in severity with time to day 7 in each frequency or treatment group, keeping the significant difference among animals treated with h-LEH, l-LEH, and animals receiving RBCs or saline; the hash symbol (#) and asterisk (*) indicate a significant difference ($P < 0.05$) from the other groups.

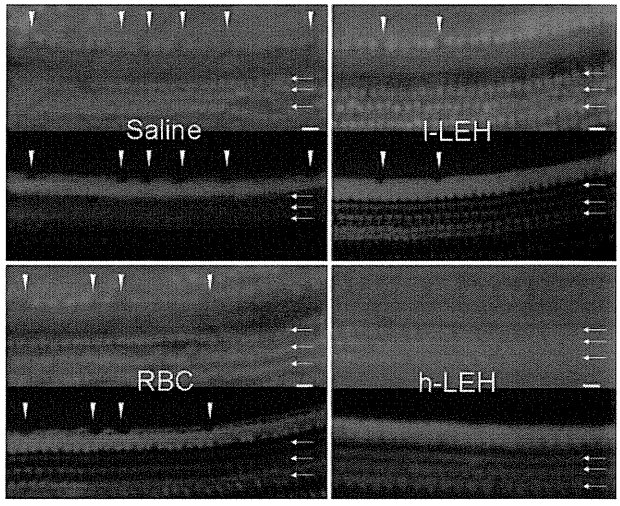

FIG. 5. While the stereocilia (rhodamine staining, red, bottom) and nuclei (Hoechst 33342, blue, top) of some IHCs had sporadically disappeared (vertical arrowheads) in the saline- and RBC-treated groups, such phenomena were less frequent in the LEH-treated groups. OHCs (horizontal arrows) remained essentially intact. Scale bar indicates 20 μm.

Morphological findings

Representative epifluorescence images of the organ of Corti from one animal in each group 7 days after ischemia (Fig. 5) showed that the stereocilia (rhodamine-phalloidin staining, red, bottom) and (Hoechst 33342 staining, blue, top) of the IHCs simultaneously disappeared, indicating cell loss. Such sporadic IHC loss contrasted sharply with the underlying OHCs, which remained mostly intact. The average ratio of dead/intact cells of IHCs on day 7 (Fig. 6) was significantly smaller in the animal groups pretreated with h-LEH ($3.7 \pm 1.2\%$, $P < 0.01$) or l-LEH ($6.5 \pm 1.2\%$, $P < 0.01$), and was less with RBCs ($15.0 \pm 2.2\%$) or saline ($14.5 \pm 2.5\%$). The latter two groups were almost equal, showing no significant difference. In contrast, cell loss was much less frequent in OHCs, with no significant difference among the treatment groups.

DISCUSSION

As acute interruption of the blood supply to the cochlea is considered to be one of the major causes of sudden deafness (1,2), remedies have been proposed to treat cochlear hypoxia. Nonetheless, widely accepted therapeutic approaches have been limited (3,4), suggesting the complex nature of the mechanism(s) involved and the variability of symptoms due in part to the differential vulnerability of the hindbrain to hypoxia (15). This led us, in the current study, to examine the effect of LEH as an artificial O_2

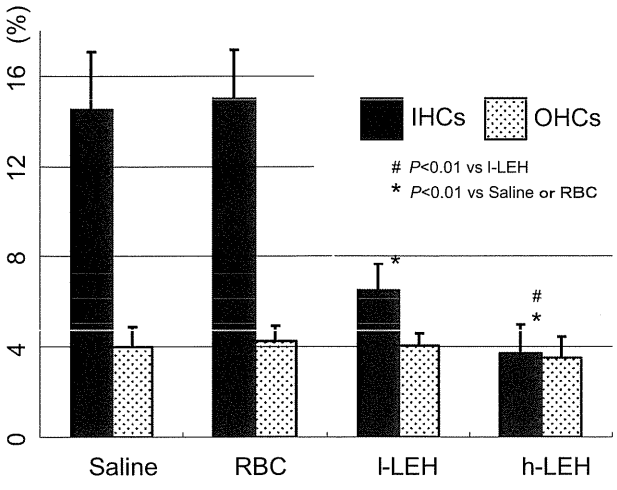

FIG. 6. Average cell loss at the basal turn 7 days after ischemia/reperfusion was more prominent in IHCs, significantly less frequent in the order of animals receiving h-LEH, l-LEH, and saline or RBCs, than in OHCs, which remained essentially intact. # $P < 0.01$ vs l-LEH; * $P < 0.01$ vs Saline or RBC.

carrier with the aim of increasing the O_2 supply, and it was revealed that the average increase in ABR threshold was significantly milder in the order of animals pretreated with h-LEH, l-LEH, and RBCs or saline. Morphologically, the disappearance of stereocilia and nuclei of IHCs in the organ of Corti was clearly correlated with the functional results, with significant differences among the treatment groups.

As the cochlea is solely perfused by the common cochlear artery as an end artery with few collaterals (18), there was severe reduction in cochlear blood flow during occlusion of the bilateral vertebral arteries (Fig. 3). Nonetheless, it remains unclear whether the blood flow was completely interrupted or persisted at least to some degree, as laser Doppler measurements reflect the average RBC flow in the area exposed to laser. In fact, plasma flow might have been different, as in the observation of Villringer et al. (19), who reported that plasma flow decreased along with RBC flow, but it still remained homogeneous and persistent even to the ischemic core. Therefore, it is plausible that LEH flows with plasma, shortens the O_2 diffusion distance, and thereby suppresses hearing defect (function) as well as IHC loss (morphology), leading to preservation of cochlear integrity. This finding is in agreement with our previous observation (16) that ABR threshold elevation caused by cochlear ischemia was due mainly to the damage to IHCs, which is considered more vulnerable to ischemia due to their high energy consumption, as compared with OHCs.

The current results disclosed that h-LEH was more effective than l-LEH in preventing ischemic and/or reperfusion injury to the inner ear. We consider that the high sensitivity and specificity of ABR allowed delineation of functional differences between treatment groups, in contrast to our previous studies that showed no apparent neurological improvements despite morphological differences (9,20). Quantitative morphometry, counting most of the IHCs in each animal, might also have allowed precise delineation of the morphological effects of each treatment. These results were in accordance with our recent observation that h-LEH provided a similar level of protection at a 1/5 to 1/25 dosage as that of l-LEH after middle cerebral artery (MCA) occlusion and reperfusion (11). As O_2 affinity is the only difference between these LEHs, the superiority of h-LEH over l-LEH may derive from the greater and more efficient O_2 delivery (Fig. 1) to tissues under hypoxic condition, or targeted O_2 delivery to ischemic cochlear tissue.

The global hindbrain ischemia induced in the current study may differ from the previous application of LEHs to focal brain ischemia (9–12), where LEH was reported to improve microcirculation to the periphery of the ischemic focus (21) or penumbral lesion, thereby eventually reducing the extent of infarction (22). Instead, the current model induced bilateral and global ischemia and reperfusion injury to the hindbrain, including the brain stem and cerebellum, which can result in respiratory arrest, convulsion, and death. For survival and long-term observation, the length of ischemia was limited to a maximum of 15 min, which was much shorter than in our previous experiments in MCA regions (9–12). This may have altered the severity of ischemia and reperfusion injury, cell survival, and apoptosis, and therefore the presentation of functional as well as morphological sequelae. Sporadic versus concentric distribution of neural cell loss may have been due to these factors. While the tendency toward functional recovery is common, the improvement was considered to be due to neural compensation rather than recovery of the affected neuronal tissues. As the development of edema may further reduce blood flow and aggravate neuronal cell loss according to the vulnerability to ischemia (15), LEH may be protective in such a global ischemia and reperfusion response (23), as it was observed in our previous study (10) that the reduction of brain edema by LEH was not limited just to the focus of ischemia in the MCA region but rather extended to the ipsilateral hippocampus and even to the pyriform lobe and contralateral hippocampus. We previously reported that

transient cochlear ischemia causes a remarkable increase in nitric oxide production in the perilymph, and that this is attributable to the inducible nitric oxide synthase (iNOS) pathway (24). As the iNOS gene includes the hypoxia-responsive element (25), oxygen delivery could alleviate reperfusion injury by decreasing iNOS gene activation. Therefore, it is possible that the antioxidant property may also be involved, may be more potent in h-LEH, and may be able to better protect ischemic tissues from reperfusion injury. Although the animals were pretreated in order to examine the maximum effects of LEHs in the current study, it is necessary to examine the efficacy of LEHs used therapeutically or even after reperfusion in order to delineate the effects on reperfusion.

CONCLUSION

The results showed that pretreatment with LEH could alleviate hearing impairment (function) as well as inner hair cell loss (morphology) for up to 7 days following transient cochlear ischemia and reperfusion. While the results suggest that more efficient O_2 delivery (l-LEH < h-LEH) during ischemia became apparent after reperfusion as in our previous experiments (9–12), the possible involvement of the antioxidant property of LEH cannot be ruled out. Therefore, timing, O_2 affinity, and dosage need to be explored during a longer-term observation to determine the effects of LEH on cochlear ischemia and reperfusion in an experimental model of sudden deafness.

Acknowledgments: We thank Arndt Gerz for the correction and refinement of the English presentation.

Authors' contributions and conflicts of interest: All the authors, except A.T. Kawaguchi and K. Imai, are clinicians/scientists (Otolaryngology) belonging to the Ehime University Graduate School of Medicine or Takanoko Hospital, who carried out the current experiments supported by grants (Grant-in-Aid for Scientific Research 20599011and 20390442 from the Ministry of Education, Culture, Science and Technology, Tokyo, Japan and a research grant from the Ministry of Health, Labor, and Welfare, Japan). A.T. Kawaguchi and K. Imai, supported by grants (Grant-in-Aid for Scientific Research 14370365, 16209037 and 20249072 from the Ministry of Education, Culture, Science and Technology, New Energy Development Organization, and a research grant from Terumo Company Limited, Tokyo, Japan), organized the experiments, discussed the results, and summarized the report with the rest of the coauthors. The LEH examined in this report was developed and supplied by Terumo Company, which received grant support (New Energy Development Organization). All authors worked in line with their own interests and grants and have no other monetary dependency to declare.

REFERENCES

1. Kim JS, Lopez I, DiPatre PL, Liu F, Ishiyama A, Balow RW. Internal auditory artery infarction: clinicopathologic correlation. *Neurology* 1999;52:40–4.
2. Fetterman BL, Saunders JE, Luxford WM. Prognosis and treatment of sudden sensorineural hearing loss. *Am J Otol* 1996;17:529–36.
3. Conlin AE, Parnes LS. Treatment of sudden sensorineural hearing loss: I. A systematic review. *Arch Otolaryngol Head Neck Surg* 2007;133:573–81.
4. Makara GB, Haller J. Non-genomic effects of glucocorticoids in neural system. Evidence, mechanisms and implications. *Prog Neurobiol* 2001;65:367–90.
5. Watanabe F, Koga K, Hakuba N, Gyo K. Hypothermia prevents hearing loss and progressive hair cell loss after transient cochlear ischemia in gerbils. *Neuroscience* 2001;102:639–45.
6. Takeda S, Hakuba N, Yoshida T, et al. Postischemic mild hypothermia alleviates hearing loss because of transient ischemia. *Neuroreport* 2008;19:1325–8.
7. Ogata Y. Characteristics and function of human hemoglobin vesicles as an oxygen carrier. *Polym Adv Technol* 2000;11: 205–9.
8. Kaneda S, Ishizuka T, Goto H, Kimura T, Inaba K, Kasukawa H. Liposome-encapsulated hemoglobin, TRM-645: current status of the development and important issues for clinical application. *Artif Organs* 2009;33:146–52.
9. Kawaguchi AT, Fukumoto D, Haida M, Ogata Y, Yamano M, Tsukada H. Liposome-encapsulated hemoglobin reduces the size of cerebral infarction in the rat: evaluation with photochemically induced thrombosis of the middle cerebral artery. *Stroke* 2007;38:1626–32.
10. Kawaguchi AT, Kurita D, Furuya H, Yamano M, Ogata Y, Haida M. Liposome-encapsulated hemoglobin alleviates brain edema after permanent occlusion of the middle cerebral artery in the rat. *Artif Organs* 2009;33:153–8.
11. Fukumoto D, Kawaguchi AT, Haida M, Yamano M, Ogata Y, Tsukada H. Liposome-encapsulated hemoglobin reduces the size of cerebral infarction in the rat: effect of oxygen affinity. *Artif Organs* 2009;33:159–63.
12. Kawaguchi AT, Haida M, Yamano M, Fukumoto D, Ogata Y, Tsukada H. Liposome-encapsulated hemoglobin ameliorates ischemic stroke in nonhuman primates: an acute study. *J Pharmacol Exp Ther* 2010;332:429–36.
13. Nogami Y, Kinoshita M, Takase B, et al. Liposome-encapsulated hemoglobin transfusion rescues rats undergoing progressive hemodilution from lethal organ hypoxia without scavenging nitric oxide. *Ann Surg* 2008;248:310–9.
14. Yoshiba F, Kawaguchi AT, Hyodo O, Kinoue T, Inokuchi S, Kato S. Possible role of artificial oxygen carrier in transfusion medicine: a retrospective analysis on current transfusion practice. *Artif Organs* 2009;33:127–32.
15. Hata R, Matsumoto M, Hatakeyama T, et al. Differential vulnerability in the hindbrain neurons and local cerebral blood flow during bilateral vertebral occlusion in gerbils. *Neuroscience* 1993;56:423–39.
16. Hakuba N, Koga K, Shudou M, Watanabe F, Mitani A, Gyo K. Hearing loss and glutamate efflux in the perilymph following transient hindbrain ischemia in gerbils. *J Comp Neurol* 2000;418:217–26.

17. Institute of Laboratory Animal Resources. *Guide for the Care and Use of Laboratory Animals*, 7th Edition. Washington, DC: Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, 1996.
18. Nakashima T, Naganawa S, Sone M, et al. Disorders of cochlear blood flow. *Brain Res Rev* 2003;43:17–28.
19. Villringer A, Them A, Lindauer U, Einhaupl K, Dirnagl U. Capillary perfusion of the rat brain cortex. An in vivo confocal microscopy study. *Circ Res* 1994;75:55–62.
20. Kawaguchi AT, Nakai K, Fukumoto D, Yamano M, Haida H, Tsukada H. *S*-nitrosylated pegylated hemoglobin reduces the size of cerebral infarction in rats. *Artif Organs* 2009;33: 183–8.
21. Urakami T, Kawaguchi AT, Akai S, et al. In vivo distribution of liposome-encapsulated hemoglobin determined by positron emission tomography. *Artif Organs* 2009;33:164–8.
22. Dirnagl U, Iadecola C, Moskowitz MA. Pathology of ischemic stroke: an integrated view. *Trends Neurosci* 1999;22:391–7.
23. Taylor JM, Crack PJ. Impact of oxidative stress on neural survival. *Clin Exp Pharmacol Physiol* 2004;31:397–406.
24. Morizane I, Hakuba N, Hyodo J, et al. Ischemic damage increases nitric oxide production via inducible nitric oxide synthase in the cochlea. *Neurosci Lett* 2005;391:62–7.
25. Kainanen R, Vartiainen N, Koistinaho J. Molecular cloning and characterization of the rat inducible nitric oxide synthase (iNOS) gene. *Gene* 1999;234:297–305.

Otology & Neurotology
32:1422–1427 © 2011, Otology & Neurotology, Inc.

Protection Against Ischemic Cochlear Damage by Intratympanic Administration of AM-111

Yoshinori Omotehara, Nobuhiro Hakuba, Naohito Hato, Masahiro Okada, and Kiyofumi Gyo

Department of Otolaryngology–Head and Neck Surgery, Ehime University, Ehime, Japan

Objective: AM-111, a cell-permeable peptide inhibitor of c-Jun N-terminal kinase, was investigated for its protective effects against ischemic damage of the cochlea in gerbils.
Methods: Transient cochlear ischemia was introduced in animals by occluding the bilateral vertebral arteries for 15 minutes. Then, 10 μl of AM-111 at a concentration of 1, 10, or 100 μM in hyaluronic acid gel formulation was applied onto the round window 30 minutes after the insult. Gel without active substance was used in a control group. Treatment effects were evaluated by auditory brainstem response (ABR) and histology of the inner ear.
Results: In controls, transient cochlear ischemia caused a 25.0 ± 5.0 dB increase in the ABR threshold at 8 kHz and a decrease of 13.3 ± 2.3% in inner hair cells at the basal turn on Day 7. Ischemic damage was mild at 2 and 4 kHz. When the animals were treated with AM-111 at 100 μM, cochlear damage was significantly reduced: the increase in ABR threshold was 3.3 ± 2.4 dB at 8 kHz, and the inner hair cell loss was 3.1 ± 0.6% at the basal turn on Day 7. The effects of AM-111 were concentration dependent: 100 μM was more effective than 1 or 10 μM.
Conclusion: Direct application of AM-111 in gel formulation on the round window was effective in preventing acute hearing loss because of transient cochlear ischemia. **Key Words:** AM-111—c-Jun N-terminal kinase—Mongolian gerbil—Prevention of hearing loss—Transient cochlear ischemia.

Otol Neurotol **32:**1422–1427, 2011.

Cochlear ischemia is considered to be one of the etiologic factors that can trigger idiopathic sudden sensorineural hearing loss (ISSNHL). We developed an animal model of transient cochlear ischemia in gerbils and studied the mechanisms of ischemia/reperfusion injury of the cochlea (1–3). In this model, acute sensorineural hearing loss was induced, mainly at higher frequencies. Histologic studies showed that the ischemic insult caused apoptotic cell death of the neural structures in the cochlea; the damage was more severe in the inner hair cells (IHCs) than in the outer hair cells (OHCs) (4,5). Using this animal model, we investigated a variety of candidate medicines for prevention of ischemia-induced hearing loss (6–10).

AM-111 is a cell-penetrating peptide that selectively inhibits the c-Jun N-terminal kinase (JNK) signaling pathway in the process of apoptotic cell death. It is prepared in a hyaluronic acid gel formulation to be placed on the round window membrane. When administered, it is transferred to virtually all sensorineural structures in the cochlea because of its TAT peptide carrier and good permeation through the round window membrane. It also possessed long pharmacologic activity due to a highly protease-resistant D-retro-inverso form (11). AM-111 has been shown to be effective in protecting hearing in the presence of various cochlear insults, such as noise trauma (11–15), acute labyrinthitis (16), cochlear implant electrode insertion trauma (17–19), aminoglycoside ototoxicity (11,18,20), and semicircular canal injury in otitis media (21). However, its effects on ischemic cochlear damage remain unclear. The purpose of the present study was to determine whether and how AM-111 can prevent ischemic damage of the cochlea by applying it on the round window.

Address correspondence and reprint requests to Yoshinori Omotehara, M.D., Department of Otolaryngology–Head and Neck Surgery, Ehime University, Shizukawa, Toon, Ehime, 791-0295, Japan; E-mail: omotehar@m.ehime-u.ac.jp

This study was financially supported by research grants from the Ministry of Health, Labor and Welfare (Acute Profound Deafness Research Committee) and from the Ministry of Education, Culture, Sports, Science and Technology, Japan (Grant-in Aid for Scientific Research (B) 20390442).

MATERIALS AND METHODS

Under approval by the ethics committee of Ehime University Graduate School of Medicine, the present study was conducted according to our institute's Guidelines for Animal Experimentation. The animals were housed in an animal room with a temperature of 21°C to 23°C and a 12/12-hour light/dark cycle (lights on: 7 AM to 7 PM). The animals had free access to food and water until the end of the experiment. All efforts were made to minimize the number of animals and suffering after the experimental protocol.

Adult 12- to 16-week-old Mongolian gerbils weighing 60 to 80 g were used. Transient cochlear ischemia was induced using a procedure described by Hata et al. (22). In Mongolian gerbils, the posterior communicating arteries of Willis' circle close spontaneously at 2 to 3 weeks after birth. Because in adults, the cochleae receive their blood supply solely from the vertebral arteries, cochlear ischemia is easily introduced by obstructing the bilateral vertebral arteries at the neck.

Anesthesia was introduced with a mixture of 3% halothane and nitrous oxide/oxygen (7:3) gas and then maintained with a mixture of 1% halothane gas. The animals were artificially ventilated using a ventilation tube inserted through the mouth. The tidal volume was set to 1 ml, and the rate was set to 70 times per minute. Body temperature was maintained at 37°C to 38°C with a heat lamp during the surgical procedure. The vertebral arteries were exposed bilaterally and dissected free from the surrounding connective tissue through a ventral transverse incision of the neck. Silk threads (4-0) were loosely looped around each artery, and the ischemia was induced on bilateral cochleae by pulling the ligatures with weights of 5 g for 15 minutes. The threads were subsequently removed to allow reperfusion, which was confirmed by observation with an operating microscope (Fig. 1). After these procedures, the otic bulla was opened to expose the round window. Next, 10 μl of AM-111 at a concentration of 1, 10, or 100 μM in a hyaluronic acid gel formulation (n = 6 for each group) was placed onto the round window membrane 30 minutes after ischemia. The gel formulation with no active substance (phosphate-buffered saline [PBS]) was used in the control group (n = 6). Finally, the wound was closed, and the animal was returned to the animal center. Separate from these studies, the effects of AM-111 in gel formulation placed onto the round window were investigated in 6 animals without induction of cochlear ischemia. This was performed to elucidate possible side effects of AM-111 by applying 100 μM of the agent. The experimental protocol was identical to that for ischemic animals.

For evaluation of auditory function, auditory brainstem responses (ABRs) were recorded using a signal processor (NEC Synax 1200, Tokyo, Japan) before and 4 and 7 days after the ischemic insult. Needle recording electrodes were placed in the vertex (reference), ipsilateral retroauricle (indifferent), and contralateral retroauricle (ground). Stimulus sounds were tone bursts of 8 kHz (0.5 ms rise/fall time), 4 kHz (1 ms rise/fall time), and 2 kHz (1 ms rise/fall time) with a plateau of 10 ms and stimulus rate of 9.5 Hz. They were applied in 10-dB steps, with 5-dB steps near the threshold. The responses were processed through a 50- to 3,000-Hz bandpass filter and averaged 300 times. Sound pressure in front of the tympanic membrane was monitored using a small microphone incorporated in a sound conduction tube. Sequential changes in hearing were assessed by comparing the thresholds of ABR before and after the insult.

Animals were sacrificed for histologic study on Day 7 after recordings of ABR. Under deep anesthesia, the animal was decapitated, and the otic bullae were removed. Each cochlea was intrascalarly perfused with 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4 and postfixed for 2 hours with the same fixative at 4°C. It was then immersed in PBS. After removal of the lateral bony wall using sharp tweezers and microscissors, the organ of Corti at the basal turn was dissected out by means of surface preparation. According to our previous study, the basal turn is most vulnerable to ischemic insult (2) and was thus the preferred site for assessment of the protective effects of the agent. The specimens were stained for 30 minutes at room temperature with rhodamine-phalloidin (Molecular Probes, Eugene, OR, USA) diluted 250 times in PBS containing 0.25% Triton C-100 and 1% bovine serum albumin. After rinsing in PBS, they were again stained with Hoechst 33342 (Calbiochem-Novabiochem Corporation, La Jolla, CA, USA), dissolved in PBS, in a dark room for 1 hour. Specimens were then rinsed in PBS and mounted in carbonate-buffered glycerol (1 part 0.5 M carbonate buffer at pH 9.5 to 9 parts glycerol) containing 2.5% 1, 4 diazabicyclo [2,2,2] octane to retard bleaching of the fluorescent signal. Fluorescence was detected using an Olympus BX60 microscope equipped with green (BP 546, FT 580, LP 590 nm) and UV (BP 365, FT 395, LP 397 nm) filters. Intact and dead hair cells were

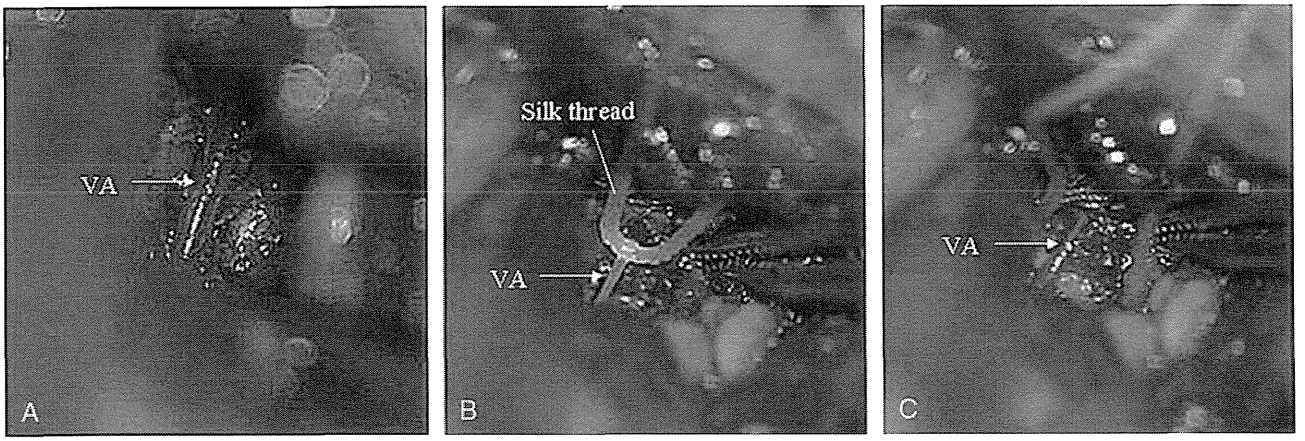

FIG. 1. Transient interruption of the cochlear blood flow. *A*, Exposure of the vertebral artery. *B*, Interruption of the cochlear blood flow by pulling silk thread looped around the artery. *C*, After loading transient ischemia, the thread was released and removed to allow recirculation.

counted, and the number of dead IHCs was calculated as a percentage of the whole number of IHCs.

The data were presented as means ± standard deviation. Statistical significance was assessed with a 1-way analysis of variance followed by Fisher's post hoc test. Values of $p < 0.05$ were considered to indicate statistical significance.

RESULTS

Transient cochlear ischemia caused hearing loss immediately after the insult, which subsequently recovered to some extent over time. Figure 2 summarizes the increases in ABR thresholds at 2, 4, and 8 kHz on Days 4 and 7, with the threshold before ischemia being defined as 0 dB. In control animals (gel formulation with PBS), the ABR threshold increase at 8 kHz was 30.8 ± 5.3 dB on Day 4, which recovered slightly on Day 7 (25.0 ± 5.0 dB). The increase was mild at other frequencies: 4.2 ± 3.8 dB at 4 kHz and 5.0 ± 4.1 dB at 2 kHz on Day 7. Ischemic cochlear damage was attenuated by administration of AM-111. In animals treated with AM-111 at 100 μM, the ABR threshold increase at 8 kHz was 6.7 ± 2.4 dB on Day 4, which further improved to 3.3 ± 2.4 dB on Day 7. When animals were treated with AM-111 at 10 or 1 μM, the increase on Day 7 was 12.5 ± 2.5 dB and 16.7 ± 3.7 dB, respectively. Statistical analysis revealed that hearing impairment at 8 kHz was prevented by AM-111 at all 3 concentrations ($p < 0.01$ at 100 and 10 μM, and $p < 0.05$ at 1 μM). Hearing impairment at 4 and 2 kHz was rather mild in this animal model, and the effects of AM-111 were not evaluated at these frequencies.

Ischemic insult causes apoptotic cell death in the organ of Corti, and the damage is more severe in the IHCs than in the OHCs (5). In controls, the percentage of IHC loss at the basal turn was 13.3 ± 2.7% on Day 7, whereas that of OHC loss was 2.4 ± 0.7%. Figure 3 summarizes the loss of IHCs at 3 turns of the cochlea. As expected, the basal turn was most affected. Administration of AM-111 at 100 μM in gel formulation was proven effective in preventing ischemic damage to the cochlea: the mean IHC loss at the basal turn was only 3.1 ± 0.6%. At lower concentrations, the protective effects of AM-111 were less pronounced: IHC loss was 5.5 ± 0.7% in animals treated with the 10-μM concentration and 9.9 ± 1.2% in those treated with AM-111 at 1 μM. The effects were statistically significant ($p < 0.01$ at 100 and 10 μM, and $p < 0.05$ at 1 μM). The effects of AM-111 on OHC loss were unclear, as the OHC damage was minor in this animal model: the percentage of OHC loss at the basal turn was 1.5 ± 0.9%, 2.6 ± 0.8%, and 3.4 ± 1.1%, when treated with 100, 10 and 1 μM of the agent, respectively. Microscopic findings of the organ of Corti at the basal turn are shown in Figure 4.

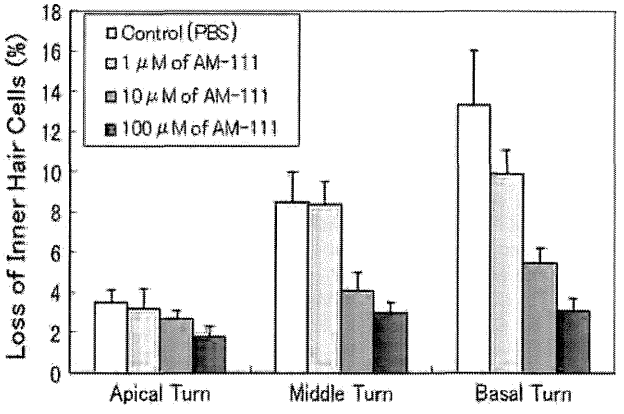

FIG. 3. Percentages of IHC loss at 3 turns of the cochlea and the effects of AM-111 in gel formulation placed on the round window. Administration of AM-111 prevented loss of IHCs at the basal turn (n = 6 for each group).

In animals without induction of cochlear ischemia, the ABR threshold increase on Day 7 after application of AM-111 at 100 μM was 0.8 ± 2.5 dB at 2, 4, and 8 kHz, respectively. These changes were minor and statistically not significant. Histologic study results also revealed that the inner ear was not affected by application of the agent.

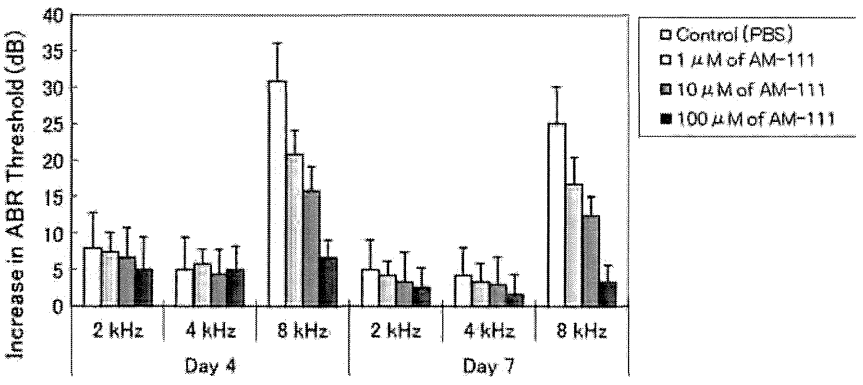

FIG. 2. ABR threshold increase after transient cochlear ischemia and the effects of AM-111 in gel formulation placed on the round window. ABR threshold before occlusion of the bilateral vertebral arteries was defined as 0 dB. Relative increases in ABR threshold at 2, 4, and 8 kHz and 1 standard deviation (n = 6 for each group) are represented. Administration of AM-111 prevented an increase in the ABR threshold, especially at 8 kHz, where it was most affected by ischemic insult. Effects of AM-111 were concentration dependent: AM-111 at 100 μM was more effective than that at 1 or 10 μM.

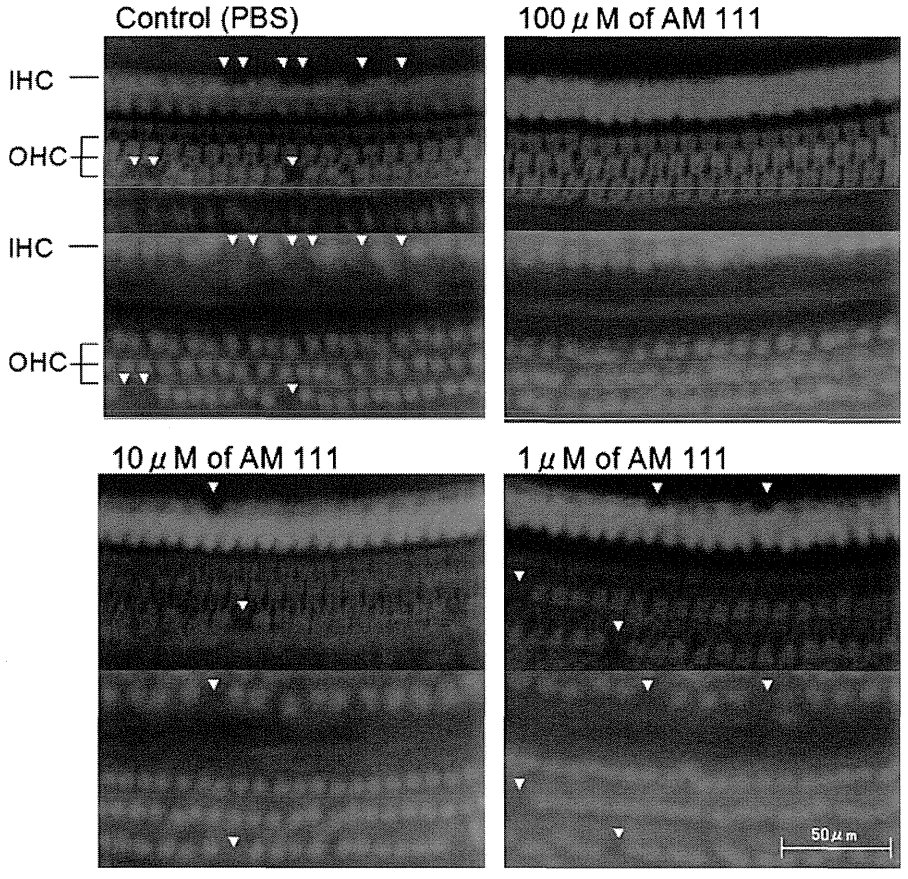

FIG. 4. Histologic findings of the cochlea at the basal turn on Day 7. *Triangles* indicate sites of hair cell loss. Rhodamine-phalloidin was used for staining cellular structures in red color and Hoechst 33342 for staining nuclei in blue color.

These findings suggest that AM-111 in gel formulation can be safely applied onto the round window without causing cochlear damage.

DISCUSSION

According to recent experimental studies, the JNK signaling pathway plays an important role in a cascade of programmed cell death (23). In the cochlea, various insults, such as loss of trophic factor (19), administration of ototoxic agents (11,18,20), and exposure to loud noise (11–15), were shown to cause apoptotic cell death of auditory neurons and hair cells through activation of the JNK pathway. AM-111, or D-JNKI-1 as it was previously named, is a novel medicine developed by Bonny et al. (24) to protect the cochlea by blocking the JNK pathway. It is now distributed for clinical and experimental studies by Auris Medical (Switzerland). The compound was effective in animal experiments in preventing neomycin ototoxicity and hearing loss due to acoustic trauma (14,15), semicircular canal injury (21), and acute labyrinthitis (16). In a study of focal cerebral ischemia, the active substance also was effective in the reduction of early calpain activation, late caspase 3 activation, and autophagosome formation, indicating involvement of the JNK pathway in 3 different types of cell death: necrosis, apoptosis, and autophagic cell death. Based on these findings, Ginet et al. (25) predicted that blocking the JNK pathway would be a novel modality in the treatment of brain ischemia in the future. In an experimental study in rats, it was further shown to be effective in preventing ischemic damage of the brain, even when administered 6 or 12 hours after the insult (26–28). The JNK inhibitor not only reduced the size of ischemic brain lesions but also lessened brain edema. Similar findings were noted in an animal model of cardiac ischemia (29).

In the present study, administration of AM-111 prevented an increase in the ABR threshold and loss of IHCs due to transient cochlear ischemia. The percentage of IHC loss caused by ischemia seemed rather great for the increase in ABR threshold. This is probably related to the cochlear anatomy, in which the IHCs are innervated much more densely than the OHCs. A single IHC is innervated by numerous nerve fibers, whereas a single nerve fiber innervates many OHCs. Correspondingly, the ABR threshold depends on whether the main lesion involves the OHC or IHC, although the hair cell damage is usually mixed. As previously stated, apoptosis is the dominant mode of cell death in this animal model (5). Various

mechanisms are supposedly involved in the process of cochlear cell death, such as excess production of free radicals, glutamate excitotoxicity, and failure of energy supply. Excess production of free radicals occurs as a process of ischemia/reperfusion injury that induces disintegration of the cell membrane through lipid peroxidation. Ischemic insult also facilitates expression of inducible nitric oxide (NO) synthase, a potent NO-synthesizing enzyme (30). Although NO is a reactive oxygen species and plays an important role in regulating vasoconstriction and neurotransmission, an excessive amount of NO leads to reactions with other molecules and increases the production of harmful free radicals, such as peroxynitrite radicals, which, in turn, cause hair cell loss by activation of JNK (14). Glutamate is a neurotransmitter released at the synapse between IHCs and primary auditory neurons. Ischemia leads to a drop in energy supply, increased depolarization, and excessive release of glutamate from the synaptic cleft, which then spreads to the surroundings (1,31). IHCs undergo apoptotic cell death as glutamate activates the JNK signaling pathway (26). This may explain why IHCs are more vulnerable to ischemic insult than OHCs, as shown by Amarjargal (32). Energy failure also causes cell death by a necrotic process. The cochlear sensory organ is sensitive to ischemic insult because in the cochlea, there is no storage of energy sources, such as glycogen. Neural structures in the inner ear use ATP as an energy source, which is produced during aerobic glycolysis. Because the glucose concentration is limited in the cochlea, existing ATP is soon exhausted by ischemia.

AM-111 is a water-soluble compound that can be intravenously administered. This is usually convenient and advantageous; however, delivery of a sufficient concentration of medicine into the cochlea is not always possible because the inner ear is protected by the blood-labyrinth barrier. Furthermore, systemic administration might cause unpredictable side effects. Alternatively, drug delivery through the round window has been proposed as an efficient way to supply pharmaceutical compounds into the cochlea. Wang et al. (13) used an osmotic mini-pump to administer AM-111 through the round window. Histologic examination showed that cellular uptake started as early as 30 minutes after AM-111 application. They concluded that the round window membrane was permeable not only to small molecules but, because of the intracellular transporter D-TAT, also to larger molecules such as the 31-amino-acid AM-111 peptide, with a molecular weight of 3820 Da. The authors also applied AM-111 in gel formulation directly on the round window. Using this procedure, the protective effect of AM-111 can be obtained with a single administration and a relatively low concentration of 100 μM.

The clinical development of AM-111 was initiated in 2006 with a prospective randomized phase I/II study in Germany by Suckfuell et al. (15). In the trial, a single dose of AM-111 at 0.4 or 2.0 mg/ml in gel formulation was administered by intratympanic injection to 11 patients with acute acoustic trauma within 24 hours after noise exposure. The results were encouraging: the pure-tone average at 4 and 6 kHz before treatment was 36 ± 16 dB, and this improved by 11 ± 12 dB after 3 days and 11 ± 14 dB after 30 days of treatment with AM-111. Currently, a much larger phase IIb clinical trial is ongoing in several European countries to treat patients with acute noise trauma or ISSNHL within 48 hours from onset. Cochlear ischemia is one of several possible origins of ISSNHL.

In conclusion, AM-111, an inhibitor of the JNK signaling pathway, can prevent ischemic damage of the cochlea when administered shortly after the incident. Application of the medicine on the round window in a gel formulation could be a promising procedure in the treatment of acute sensorineural hearing loss triggered not only by loud noise, surgical trauma, and ototoxic agents but also by cochlear ischemia.

Acknowledgment: AM-111 was supplied courtesy of Dr. Thomas Meyer of Auris Medical.

REFERENCES

1. Hakuba N, Gyo K, Yanagihara N, Mitani A, Kataoka K. Efflux of glutamate into the perilymph of the cochlea following transient ischemia in the gerbil. *Neurosci Lett* 1997;230:69–71.
2. Koga K, Hakuba N, Watanabe F, Shudou M, Nakagawa T, Gyo K. Transient cochlear ischemia causes delayed cell death in the organ of Corti: an experimental study in gerbils. *J Comp Neurol* 2003;456:105–11.
3. Watanabe F, Hakuba N, Gyo K. Measurement of DPOAE after ischemia/reperfusion injury of the cochlea in gerbils. *Neurosci Lett* 2009;467:135–8.
4. Hakuba N, Koga K, Shudou M, Watanabe F, Mitani A, Gyo K. Hearing loss and glutamate efflux in the perilymph following transient hindbrain ischemia in gerbils. *J Comp Neurol* 2000;418:217–26.
5. Taniguchi M, Hakuba N, Koga K, Watanabe F, Hyodo J, Gyo K. Apoptotic hair cell death after transient cochlear ischemia in gerbils. *Neuroreport* 2002;13:2459–62.
6. Hakuba N, Matsubara A, Hyodo J, et al. AMPA/kainate-type glutamate receptor antagonist reduces progressive inner hair cell loss after transient cochlear ischemia. *Brain Res* 2003;979:194–202.
7. Maetani T, Hakuba N, Taniguchi M, Hyodo J, Shimizu Y, Gyo K. Free radical scavenger protects against inner hair cell loss after cochlear ischemia. *Neuroreport* 2003;14:1881–4.
8. Fujita K, Hakuba N, Hata R, et al. Ginsenoside Rb1 protects against damage to the spiral ganglion cells after cochlear ischemia. *Neurosci Lett* 2007;415:113–7.
9. Fujiwara T, Hato N, Nakagawa T, et al. Insulin-like growth factor 1 treatment via hydrogels rescue cochlear hair cells from ischemic injury. *Neuroreport* 2008;19:1585–8.
10. Maetani T, Hyodo J, Takeda S, Hakuba N, Gyo K. Prednisolone prevents transient ischemia-induced cochlear damage in gerbils. *Acta Otolaryngol* 2009;562:24–27.
11. Wang J, Van De Water TR, Bonny C, de Ribaupierre F, Puel JL, Zine A. A peptide inhibitor of c-Jun N-terminal kinase protects against both aminoglycoside and acoustic trauma-induced auditory hair cell death and hearing loss. *J Neurosci* 2003;23:8596–7.
12. Pirvola U, Xing-Qun L, Virkkala J, et al. Rescue of hearing, auditory hair cells, and neurons by CEP-1347/KT7515, an inhibitor of c-Jun N-terminal kinase activation. *J Neurosci* 2000;20:43–50.
13. Wang J, Ruel J, Ladrech S, Bonny C, van de Water TR, Puel JL. Inhibition of the c-Jun N-terminal kinase-mediated mitochondrial cell death pathway restores auditory function in sound-exposed animals. *Mol Pharmacol* 2007;71:654–66.
14. Coleman JKM, Littlesunday C, Jackson R, Meyer T. AM-111

protects against permanent hearing loss from impulse noise trauma. *Hear Res* 2007;226:70–8.
15. Suckfuell M, Canisi S, Striethi S, Scherer H, Haisch A. Intratympanic treatment of acute acoustic trauma with a cell-permeable JNK ligand: a prospective randomized phase I/II study. *Acta Otolaryngol* 2007; 127:938–42.
16. Barkdull GC, Hondarrague Y, Meyer T, Harris JP, Keithley EM. AM-111 reduces hearing loss in a guinea pig model of acute labyrinthitis. *Laryngoscope* 2007;117:2174–82.
17. Eshraghi AA, He J, Mou CH, et al. D-JNKI-1 treatment prevents the progression of hearing loss in a model of cochlear implantation trauma. *Otol Neurotol* 2006;27:504–11.
18. Eshraghi AA, Wang J, Adil E, et al. Blocking c-Jun-N-terminal kinase signaling can prevent hearing loss induced by both electrode insertion trauma and neomycin ototoxicity. *Hear Res* 2007;226: 168–77.
19. Scarpidis U, Madnani D, Shoemaker C, et al. Arrest of apoptosis in auditory neurons: implications for sensorineural preservation in cochlear implantation. *Otol Neurotol* 2003;24:409–17.
20. Ylikoski J, Xing-Qun L, Virkkala J, Pirvola U. Blockade of c-Jun N-terminal kinase pathway attenuates gentamicin-induced cochlear and vestibular hair cell death. *Hear Res* 2002;166:33–43.
21. Grindal TC, Sampson EM, Antonelli PJ. AM-111 prevents hearing loss from semicircular canal injury in otitis media. *Laryngoscope* 2010;120:178–82.
22. Hata R, Matsumoto M, Kitagawa K, et al. A new gerbil model of hindbrain ischemia by extracranial occlusion of the lateral ertebral arteries. *J Neurol Sci* 1994;121:79–89.
23. Tournier C, Hess P, Yang DD, et al. Requirement of JNK for stress-induced activation of the cytochrome c-mediated death pathway. *Science* 2000;288:870–4.
24. Bonny C, Oberson A, Negri S, et al. Cell-permeable peptide inhibitors of JNK: novel blockers of beta-cell death. *Diabetes* 2001; 50:77–82.
25. Ginet V, Puyal J, Magnin G, Clarke PG, Truttmann AC. Limited role of the c-Jun N-terminal kinase pathway in a neonatal rat model of cerebral hypoxia-ischemia. *J Neurochem* 2009;108:552–62.
26. Liu JR, Zhao Y, Patzer A, et al. The JNK-inhibitor XG-102 enhances the neuroprotection of hyperbaric oxygen after cerebral ischaemia in adult rats. *Neuropathol Appl Neurobiol* 2010;36: 211–24.
27. Repici M, Centeno C, Tomasi S, et al. Time-course of c-Jun N-terminal kinase activation after cerebral ischemia and effect of D-JNKI1 on c-Jun and caspase-3 activation. *Neuroscience* 2007;150: 40–9.
28. Borsello T, Clarke PG, Hirt L, et al. A peptide inhibitor of c-Jun N-terminal kinase protects against excitotoxicity and cerebral ischemia. *Nat Med* 2003;9:1180–6.
29. Milano G, Morel S, Bonny C, et al. A peptide inhibitor of c-Jun NH2-terminal kinase reduces myocardial ischemia-reperfusion injury and infarct size in vivo. *Am J Physiol Heart Circ Physiol* 2007;292:1828–35.
30. Morizane I, Hakuba N, Hyodo J, et al. Ischemic damage increases nitric oxide production via inducible nitric oxide synthase in the cochlea. *Neurosci Lett* 2005;391:62–7.
31. Hyodo J Hakuba N, Hato N, et al. Glutamate agonist causes irreversible degeneration of inner hair cells. *Neuroreport* 2009;20: 1255–9.
32. Amarjargal N, Andreeva N, Gross J, et al. Differential vulnerability of outer and inner hair cells during and after oxygen-glucose deprivation in organotypic cultures of newborn rats. *Physiol Res* 2009;58:895–902.

Biochemical and Biophysical Research Communications 415 (2011) 94–98

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Protective role of Nrf2 in age-related hearing loss and gentamicin ototoxicity

Tomofumi Hoshino [a], Keiji Tabuchi [a,*], Bungo Nishimura [a], Shuho Tanaka [a], Masahiro Nakayama [a], Tetsuro Ishii [b], Eiji Warabi [b], Toru Yanagawa [c], Ritsuku Shimizu [d], Masayuki Yamamoto [e], Akira Hara [a]

[a] Department of Otolaryngology, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tsukuba, Japan
[b] Institute of Community Medicine, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tsukuba, Japan
[c] Department of Oral and Maxillofacial Surgery, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tsukuba, Japan
[d] Department of Molecular Hematology, Tohoku University Graduate School of Medicine, Sendai, Japan
[e] Department of Medical Biochemistry, Tohoku University Graduate School of Medicine, Sendai, Japan

ARTICLE INFO

Article history:
Received 3 October 2011
Available online 12 October 2011

Keywords:
NF-E2-related factor (Nrf2)
Reactive oxygen species (ROS)
Cochlea
Age-related hearing loss (AHL)
Gentamicin ototoxicity

ABSTRACT

Expression of antioxidant enzymes is regulated by transcription factor NF-E2-related factor (Nrf2) and induced by oxidative stress. Reactive oxygen species contribute to the formation of several types of cochlear injuries, including age-related hearing loss and gentamicin ototoxicity. In this study, we examined the roles of Nrf2 in age-related hearing loss and gentamicin ototoxicity by measuring auditory brainstem response thresholds in *Nrf2*-knockout mice. Although *Nrf2*-knockout mice maintained normal auditory thresholds at 3 months of age, their hearing ability was significantly more impaired than that of age-matched wild-type mice at 6 and 11 months of age. Additionally, the numbers of hair cells and spiral ganglion cells were remarkably reduced in *Nrf2*-knockout mice at 11 months of age. To examine the importance of Nrf2 in protecting against gentamicin-induced ototoxicity, 3-day-old mouse organ of Corti explants were cultured with gentamicin. Hair cell loss caused by gentamicin treatment was enhanced in the *Nrf2*-deficient tissues. Furthermore, the expressions of some *Nrf2*-target genes were activated by gentamicin treatment in wild-type mice but not in *Nrf2*-knockout mice. The present findings indicate that Nrf2 protects the inner ear against age-related hearing injuries and gentamicin ototoxicity by up-regulating antioxidant enzymes and detoxifying proteins.

1. Introduction

The antioxidant responsive element (ARE) is a *cis*-acting regulatory element, through which Nrf2 regulates transcription of genes encoding phase II detoxification enzymes, antioxidants, and other factors essential for cell survival. Under normal conditions, Nrf2 is anchored in the cytoplasm through interaction with Kelch-like ECH-associated protein 1 (Keap1) and subsequently proteolyzed by proteasomes. In contrast, under oxidative stress conditions, Keap1-censored electrophiles inhibit the proteolysis of Nrf2. Having thus escaped Keap1-mediated proteolysis, Nrf2 accumulates in the nucleus and activates ARE-mediated gene transcription [1,2]. To date, many genes driven by Nrf2, including heme oxygenase 1 (HO1), NAD(P)H:quinone oxidoreductase 1 (NQO1), NHR:quinone oxidoreductase 2 (NQO2), glutathione peroxidase (GPx), superoxide dismutase 1 (SOD1), and peroxiredoxin I (PrxI), have been reported to be involved in the antioxidant defense system [3–5].

Accumulated evidence suggests that reactive oxygen species (ROS) are involved in the pathogenesis of a wide range of cochlear injuries, including cochlear ischemia–reperfusion injury [6–9], acoustic injury [10–12], aminoglycoside ototoxicity [13], and age-related hearing loss (AHL) [14]. Because ROS are one of the Nrf2-inducing stressors, Nrf2–ARE responses are predicted to give rise to cochlear injuries. However, little is known about the protective role of Nrf2 in the cochlear pathophysiology.

In contrast, several contributions of Nrf2-driven antioxidant enzymes to the cochlea have been revealed. For example, SOD1 is one of the best-characterized enzymes in the cochlear pathology. Age-related hearing loss was accelerated in *SOD1*-knockout (KO) mice [15,16], and *SOD1*-KO mice are more susceptible to noise-induced hearing loss [17]. Acoustic injury was also reported to be prevented by tempol, a SOD-mimetic agent [11]. Furthermore, HO1 and GPx effectively work to prevent inner ear injury after exposure to oxidative stresses [18–21]. Since PrxI scavenges hydrogen peroxide, lipid hydroperoxide, and peroxynitrite, PrxI is thought to contribute to the prevention of oxidative injury in the cochlea.

Here, we examined the contribution of Nrf2 to cochlear protection by using *Nrf2*-KO mice. The results from the auditory brainstem

* Corresponding author. Address: Department of Otolaryngology, Graduate School of Comprehensive Human Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba 305-8575, Japan. Fax: +81 298 53 3147.
E-mail addresses: tomofumi@md.tsukuba.ac.jp (T. Hoshino), ktabuchi@md.tsukuba.ac.jp (K. Tabuchi).

0006-291X/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2011.10.019

response (ABR) and morphological analyses indicated significant impairment of auditory function in aged mice in the absence of Nrf2. Furthermore, the cochlear cells of *Nrf2*-KO mice were severely injured after gentamicin treatment, to the extent that they lacked the expression of several *Nrf2*-driven antioxidant enzymes. These analyzes of *Nrf2*-KO mice provide new insight into preventive medical procedures for age-related and drug-induced hearing loss.

2. Materials and methods

2.1. Animals

Nrf2-KO mice were produced and maintained on a BDF1 background as previously described [2]. Wild-type BDF1 mice were used as the control. The animals were maintained under a normal day/night cycle and given free access to food and water. The care and use of animals were approved by the Animal Research Committee and Safety Committee for Gene Recombination Research of the University of Tsukuba.

2.2. ABR testing

Mice were anesthetized with an intraperitoneal injection of pentobarbital sodium (Nembutal; Abbott Laboratories, Chicago, IL). Anesthesia was supplemented during the course of measurement of ABR as necessary.

Positive, negative, and ground electrodes were subcutaneously inserted at the vertex, mastoid, and back, respectively [11,12,22]. Bursts of 4, 8, or 16 kHz pure tones were used to evoke ABR. Evoked responses were amplified, filtered with a bandpass of 200 Hz to 3 kHz, and averaged with 500 sweeps using a signal processor (Synax2100; NEC, Tokyo, Japan). The visual detection threshold was determined with increment or decrement of sound pressure at 5 dB steps.

2.3. Staining of hair cells

Eleven-month-old mice were transcardially perfused with 4% paraformaldehyde under deep anesthesia with pentobarbital sodium. After decapitation, the cochleae were kept in the same fixative at 4 °C overnight, decalcified with ethylenediaminetetraacetic acid (EDTA), and then dissected for surface preparation. Whole mounts of the organ of Corti were permeabilized with 5% TritonX-100 (Sigma, St. Louis, MO) in phosphate-buffered saline (PBS) with 10% fetal bovine serum (FBS) for 10 min. The specimens were stained with a rhodamine–phalloidin probe (1:100; Invitrogen, Carlsbad, CA) at room temperature for 1 h [23]. Phalloidin specifically binds to cellular F-actin and is used to visualize the stereociliary arrays and cuticular plate of hair cells.

2.4. Assessment of hair cell damage

A hair cell was characterized as "missing" if neither the stereocilia nor the cuticular plate were observed in the cell by phalloidin staining. Quantitative results were obtained by evaluating 30 outer hair cells associated with 10 inner hair cells in a given microscopic field. The average of five separate counts was used to represent each specimen [23,24].

2.5. Assessment of spiral ganglion cells

The *Nrf2*-KO and wild-type mice were killed at 11 months of age. The cochleae were fixed with 4% paraformaldehyde, decalcified with EDTA, and then embedded in paraffin. The cochleae were cut into 5-μm-thick midmodiolar sections. After the sections were stained with hematoxylin and eosin, the number of spiral ganglion cells was counted.

2.6. Culture techniques

The basal turn of the organ of Corti was dissected from the mice on postnatal day 3 (P3). The methods for culture of the organ of Corti were previously reported [23]. The explants were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, and 30 U/mL penicillin and cultured in an incubator at 37 °C with 5% CO_2 and 95% humidity with or without gentamicin treatment at a concentration of 50 μm. For the morphological analysis, after 72 h of culture, the explants were fixed with 4% paraformaldehyde in PBS for 20 min and then permeabilized with 5% TritonX-100 in PBS with 10% FBS for 10 min [23,24]. The specimens were stained with a rhodamine–phalloidin probe, as described above.

2.7. RT-PCR

Dissected cochleae of P3–5 pups were cultured in DMEM media as described above for up to 24 h. Total RNAs were isolated from the culture specimens using an Isogen RNA preparation kit (Nippon Gene, Tokyo, Japan). The cDNA samples were synthesized with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). qPCR Mastermix (Nippon Gene, Tokyo, Japan) was used for the analyzes of the *HO1* and *NQO1* genes and a Power SYBR Green RNA-to-CT 1 step kit (Applied Biosystems, Carlsbad, CA) for the *SOD1* and *PrxI* genes according to the manufacturer's instructions. Real-time RT-PCR was performed using an ABI-PRISM 7700 Sequence Detector System (Applied Biosystems, Carlsbad, CA). The primers were as follows, and otherwise were described previously [25]: (*SOD1* forward: 5′-GACAAACCTGAGCCCTAAG-3′; *SOD1* reverse: 5′-CGACCTTGCTCCTTATTG-3′; *PrxI* forward: 5′-CGTTCTCACGGCTCTTTCTGT3′; *PrxI* reverse:5′-GCATTTCCTGAAGACATCTTGCT-3′).

2.8. Statistical analyses

All data were evaluated by the *t* test or two-way ANOVA. Any test resulting in a *p* value of less than 0.05 was considered significant. Error bars represent the standard error of means.

3. Results

3.1. Hearing ability deteriorates rapidly with increasing age in Nrf2-KO mice

To elucidate the contribution of Nrf2 to the auditory organ, we examined hearing thresholds in *Nrf2*-KO mice by measuring the ABR thresholds. The ABR thresholds in the *Nrf2*-KO and age-matched wild-type mice at 3 months of age were not significantly different (Fig. 1). Next, to elucidate age-induced changes in hearing ability, we measured the ABR thresholds of the mice at 6 and 11 months of age. Expectedly, the ABR thresholds of the wild-type mice were elevated in an age-dependent manner (Fig. 1). Notably, the ABR thresholds of the 6- and 11-month-old *Nrf2*-KO mice rose to much higher levels than those of the age-matched wild-type mice (Fig. 1). These results indicated that Nrf2 was involved in maintaining hearing function in older mice.

3.2. Hair cells and spiral ganglion cells are missing in aged Nrf2-KO mice

To elucidate the reason for the hearing disturbance in older *Nrf2*-KO mice, the cochlear morphology was microscopically exam-

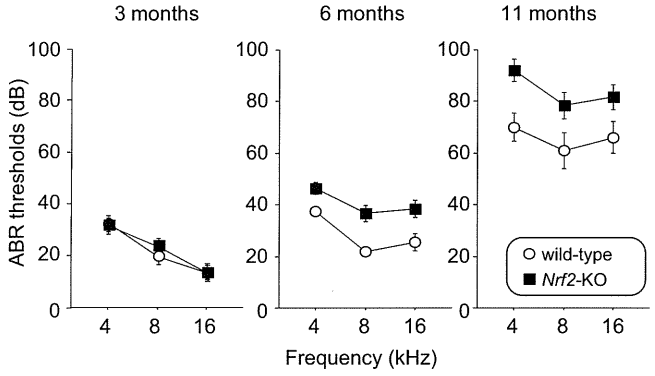

Fig. 1. ABR thresholds of *Nrf2*-KO and wild-type mice at 3, 6, and 11 months of age. The ABR thresholds in the *Nrf2*-KO mice at 6 and 11 months of age were significantly more elevated than those in the age-matched wild-type mice ($p < 0.01$, two-way ANOVA), while the *Nrf2*-KO mice showed a similar ABR pattern to that of the wild-type mice at 3 months of age. Ten wild-type and 10 *Nrf2*-KO mice at 3 months of age, 10 wild-type and 6 *Nrf2*-KO mice at 6 months of age, and 10 wild-type and 12 *Nrf2*-KO mice at 11 months of age were used for this experiment.

ined and phalloidin staining used to observe the hair cells. As shown in Fig. 2A, degeneration of the outer hair cells was apparent in the first cochlear region, while hair cell loss in the second turn and the region of inner hair cells was not obvious in the 11-month-old wild-type mice (Fig. 2A), consistent with previous reports. In contrast, the cochlear architecture of the *Nrf2*-KO mice was destroyed in the outer hair cell layer, and the hair cell loss extended into the inner hair cell layer of the second turn (Fig. 2A). We next attempted quantitative analyses of the hair cells. In good agreement with the morphological analyses, the number of residual outer hair cells was significantly more decreased in the *Nrf2*-KO mice than in the age-matched wild-type mice ($p < 0.001$, two-way ANOVA;) (Fig. 2B, upper panel). In addition, significant inner hair cell loss in the first turn of the *Nrf2*-KO mouse cochlea was also observed ($p < 0.05$, two-way ANOVA) (Fig. 2B, lower panel).

Next, the ganglion cell population in the midmodiolar sections in the 11-month-old *Nrf2*-KO and wild-type mice was counted. Microphotographs of the lower first and lower second turns of these mice showed spiral ganglion cells in the Rosenthal canal (Fig. 3A). Loss of spiral ganglion cells in the lower turn of the cochlea was clearly observed in both the wild-type and the *Nrf2*-KO mice (Fig. 3A). On the other hand, spiral ganglion cell density in the upper turn was also decreased in the *Nrf2*-KO mice, whereas it was comparatively maintained in the wild-type mice (Fig. 3A). Quantitative analysis of the spiral ganglion cells in each turn revealed that the number of residual spiral ganglion cells was significantly more decreased in the *Nrf2*-KO mice than in the wild-type mice ($p < 0.05$, two-way ANOVA). Taken together, hair cell loss and spiral ganglion cell loss rapidly progressed with age in *Nrf2*-KO mice, causing significant hearing impairments in older mice.

3.3. *Nrf2* knockout accelerated gentamicin ototoxicity

Nrf2 regulates expression of genes that protect cells from oxidative damage. To investigate the roles of Nrf2 in protection against oxidative stress in the cochlea, we attempted explant organ culture in the presence of gentamicin. Gentamicin is a well-known ototoxic drug, and its ototoxicity is involved in the ROS- and nitric oxide-related mechanisms.

Hair cell explants were dissected from P3 pups of *Nrf2*-KO and wild-type mice. Subsequently, the explants were incubated in 50 μm gentamicin for 72 h. As shown in Fig. 4A, the outer hair cells were severely damaged in the *Nrf2*-KO mice. Quantitative analysis revealed that the number of residual outer hair cells was much

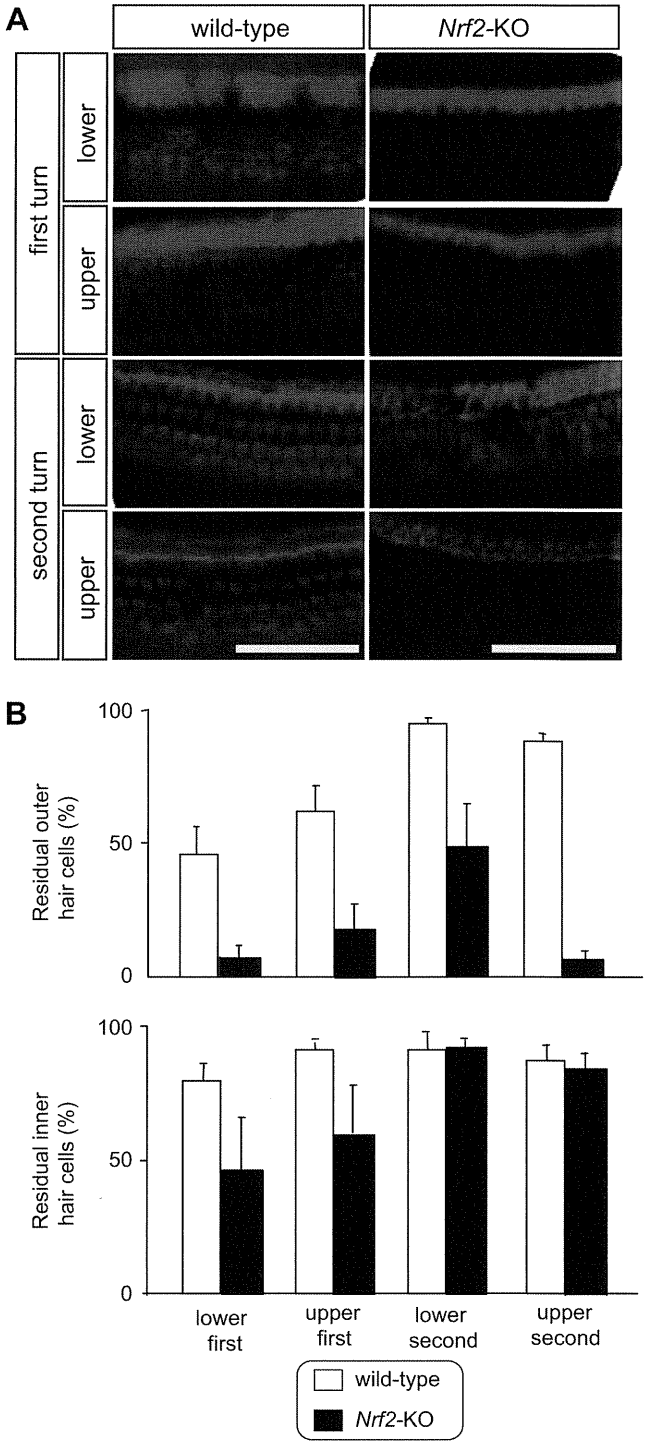

Fig. 2. Age-related degradation of cochlear hair cells in *Nrf2*-KO mice. (A) Representative sections through the organ of Corti showing inner and outer hair cells of wild-type and *Nrf2*-KO mice at 11 months of age. Bar: 50 μm. (B) Quantitative analyses of the number of residual outer and inner hair cells in mice at 11 months of age. Note the severe outer hair cell loss in the *Nrf2*-KO mice not only in the first but also in the second turn ($p < 0.01$, two-way ANOVA). The hair cell loss extended into the inner hair cell region in the first turn of the *Nrf2*-KO mice ($p < 0.01$, two-way ANOVA). Six or seven mice in each group were used for this experiment.

more decreased in the *Nrf2*-KO mice than in the wild-type mice ($p < 0.01$, *t* test) (Fig. 4B).

We next examined the expression of Nrf2-dependent enzymes in the cultured cochleae exposed to gentamicin. As shown in

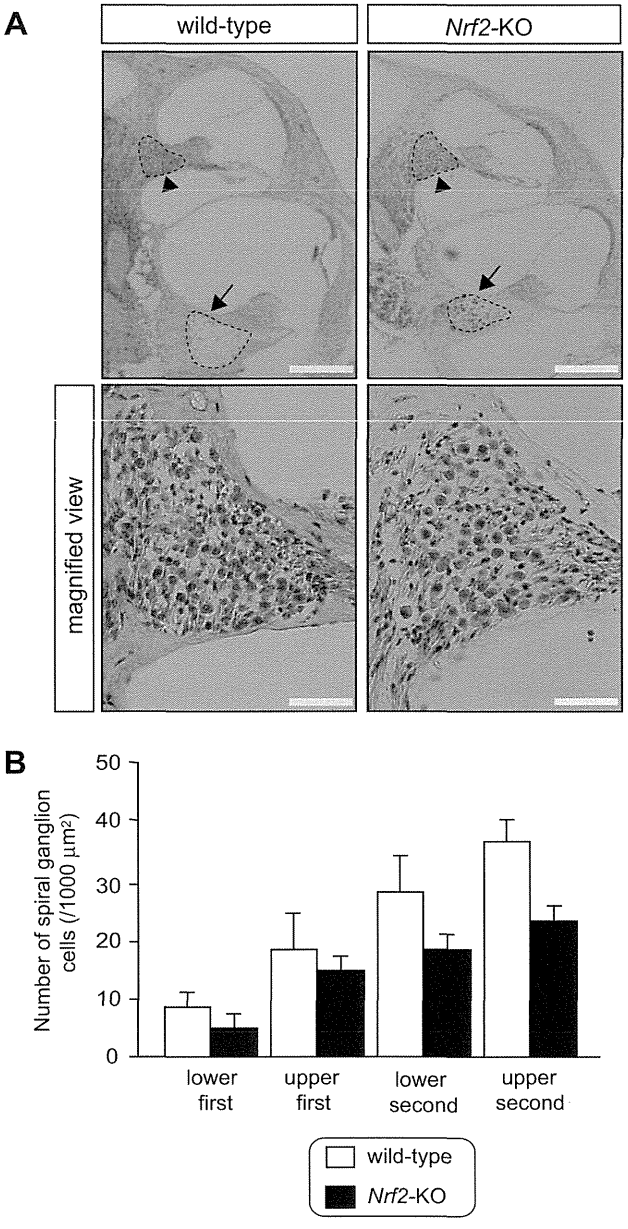

Fig. 3. Age-related spiral ganglion cell loss in *Nrf2*-KO mice. (A) Representative sections through the organ of Corti showing spiral ganglion cells of the wild-type and *Nrf2*-KO mice at 11 months of age. The areas of the Rosenthal canal in the lower first turn (arrows) and the lower second turn (arrowheads) are indicated by dotted lines. Bar: upper panel 100 μm; lower panel 20 μm. (B) Quantitative analyses of the number of residual spiral ganglion cells. The residual spiral ganglion cell population was significantly more decreased in the *Nrf2*-KO mice than in the wild-type mice ($p < 0.05$, two-way ANOVA). Five mice in each group were used for this experiment.

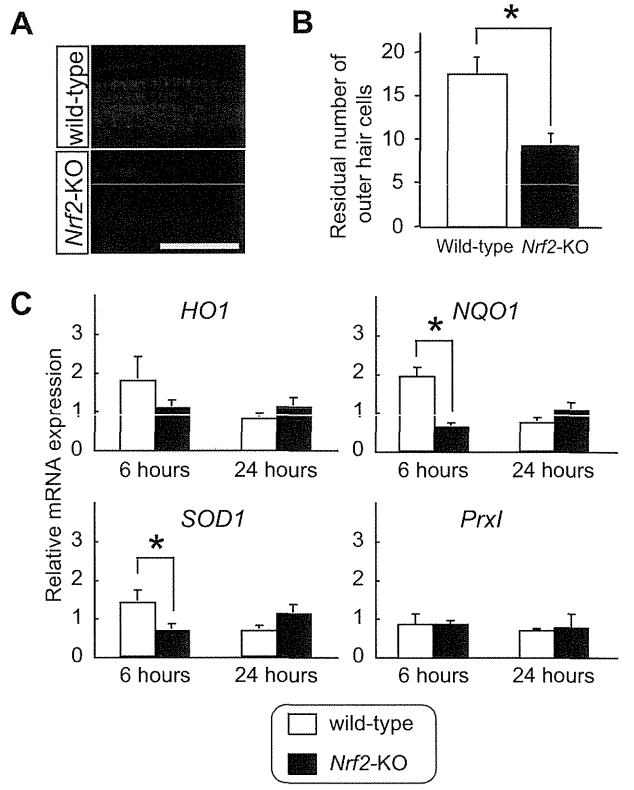

Fig. 4. Gentamicin ototoxicity in the cultured cochlear hair cells. (A) Representative sections of hair cell explants exposed to gentamicin for 72 h. The hair cells of the *Nrf2*-KO mice were more damaged than those of the wild-type mice. Bar: 50 μm. (B) Quantitative analysis of the number of residual outer hair cells after gentamicin exposure. The number of residual outer hair cells in the *Nrf2*-KO mice was significantly more reduced than that in the wild-type mice ($p < 0.01$, t test). Ten wild-type and 15 *Nrf2*-KO mice were used for this experiment. (C) Expression levels of genes coding for *Nrf2*-driven antioxidant enzymes after gentamicin exposure. The vertical axes indicate the relative ratio of the gene expression in the explants exposed to gentamicin to that in the untreated explants. Four wild-type and four *Nrf2*-KO mice were used for this experiment.

Fig. 4C, the mRNA levels of *HO1*, *NQO1*, and *SOD1* in the wild-type mice were increased at 6 h and had recovered to the basal level at 24 h after gentamicin exposure. On the other hand, such induction was not observed in the *Nrf2*-KO mice during the observation period. Especially, the expression of *NQO1* and *SOD1* genes after 6-h exposure was significantly more elevated in the wild-type mice than in the *Nrf2*-KO mice ($p < 0.05$, t test). In contrast, induction by gentamicin of *Prxl* gene expression was not detected in either the wild-type mice or the *Nrf2*-KO mice during this experiment. These findings suggest that Nrf2 protects the cochlear from gentamicin ototoxicity through induction of antioxidant enzymes.

4. Discussion

Reactive oxygen species are involved in a wide range of cochlear injuries [6–14]. In addition, AHL is ascribed to the production of ROS, at least in part [26]. Nrf2 is believed to protect against oxidative tissue damage through ARE-mediated transcriptional activation of several phase II detoxifying enzymes and antioxidant enzymes. However, little is known about the function of Nrf2 in the cochlea. So et al. demonstrated the roles of Nrf2 in the protection of cisplatin-induced hair cell loss via *HO1* gene activation by means of an *ex vivo* organ culture assay [20]. Furthermore, recent work using mice suffering from progressive hearing loss demonstrated that sulforaphane, an Nrf2 inducer, was effective in alleviating the mouse phenotype [27]. Hence, the molecular dissection of the Nrf2 function in the cochlea has become very important for the development of preventive strategies for patients at high risk of ear damage. In this study, we clarified the importance of Nrf2 in the cochlea in protecting against AHL and ototoxic agents by using genetically manipulated *Nrf2*-KO mice.

The spiral shape of the cochlea was not disturbed in *Nrf2*-KO mice. In addition, the hearing ability of these mice was within normal levels until 3 months of age. In contrast, the hearing ability of *Nrf2*-KO mice rapidly worsened with age in comparison with that of wild-type mice and was accompanied by significant loss of hair cells and spiral ganglion cells. Therefore, we surmise that Nrf2 is

preventive against progression of AHL, while loss of Nrf2 is dispensable during auditory organ development.

Aminoglycoside antibiotics, such as gentamicin, are ototoxic drugs damaging to cochlear hair cells. As in cisplatin-induced ototoxicity, ROS are one of the leading factors involved in aminoglycoside-induced cochlear injury. We here demonstrated that Nrf2 protects against progression of gentamicin-induced hair cell damage by regulating antioxidant enzymes. The expression of *NQO1* and *SOD1*, which are representative ARE-dependent Nrf2-mediated detoxifying/antioxidant enzymes, was significantly upregulated after 6-h gentamicin exposure in wild-type mice, but such upregulation was not observed in *Nrf2*-KO mice. We surmise that in a similar manner to these two genes, HO1 is cooperatively involved in otoprotection, since it is one of the important Nrf2-mediated enzymes against oxidative stress-induced inner ear injury. We could not detect any significant difference in *HO1* gene induction by gentamicin exposure between the wild-type and *Nrf2*-KO mice, probably because the expression of HO1 is regulated by a combination of Nrf2 and Bach1, which is another b-Zip protein Bach1 [28]. Further analysis using *Nrf2*- and *Bach1*-double KO mice is required to clarify the regulation of HO1 in the cochlea by gentamicin exposure. It is noteworthy that the expression of such Nrf2-target genes was increased in a transient manner when the induction response was complete by 24 h. This result is somewhat unexpected because the induction of Nrf2 target genes by conventional Nrf2-inducers usually continues beyond 24 h. It was previously reported that a transient response by diethyl maleate, a strong Nrf2 inducer, was observed in *HO1* but not in *NQO1* gene expression because of a gene-specific epigenetic modification [29]. We speculate that a variety of modification mechanisms divers the gene regulation profiles depending on the tissue identity.

In conclusion, the present study demonstrated that disruption of Nrf2 causes AHL and renders the cochlea vulnerable to gentamicin ototoxicity. Although Nrf2 does not contribute to the formation of the inner ear in the developmental period, it cleans up the ROS induced by several postbirth environmental factors, thereby minimizing AHL-related cochlear injuries. We propose that compounds that activate Nrf2 prevent progression of cochlear injury related to AHL and ototoxic agents.

Acknowledgment

This work was supported by a Grant-in-aid for Young Scientists (B) (22791567) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

References

[1] K. Itoh, T. Ishii, N. Wakabayashi, M. Yamamoto, Regulatory mechanisms of cellular response to oxidative stress, Free Radic. Res. 31 (1999) 319–324.
[2] K. Itoh, N. Wakabayashi, Y. Katoh, T. Ishii, T. O'Connor, M. Yamamoto, Keap1 regulates both cytoplasmic-nuclear shuttling and degradation of Nrf2 in response to electrophiles, Genes Cells 8 (2003) 379–391.
[3] T. Ishii, K. Itoh, S. Takahashi, H. Sato, T. Yanagawa, Y. Katoh, S. Bannai, M. Yamatomo, Transcriptional factor Nrf2 coordinately regulates a group of oxidative stress-inducible genes in macrophages, J. Biol. Chem. 275 (2000) 16023–16029.
[4] G. Schreibelt, J. Van Horssen, S. van Rossum, C.D. Dijkstra, B. Drukarch, H.E. de Vries, Therapeutic potential and biological role of endogenous antioxidant enzymes in multiple sclerosis pathway, Brain Res. Rev. 56 (2007) 322–330.
[5] J.A. Johnson, D.A. Johnson, A.D. Kraft, M.J. Calkins, R. Jakel, M.R. Vargas, P.C. Chen, The Nrf2–ARE pathway. An indicator and modulator of oxidative stress in neurodegeneration, Ann. N. Y. Acad. Sci. 1147 (2008) 61–69.
[6] K. Tabuchi, Z. Ito, T. Wada, A. Hara, J. Kusakari, The effect of mannitol upon cochlear dysfunction induced by transient local anoxia, Hear. Res. 126 (1998) 28–36.
[7] K. Tabuchi, J. Kusakari, Z. Ito, K. Takahashi, T. Wada, A. Hara, Effect of nitric oxide synthase inhibitor on cochlear dysfunction induced by transient local anoxia, Acta Otolaryngol. 119 (1999) 179–184.
[8] K. Tabuchi, H. Okubo, K. Fujihira, S. Tsuji, A. Hara, J. Kusakari, Protection of outer hair cells from reperfusion injury by an iron chelator and a nitric oxide synthase inhibitor in the guinea pig cochlea, Neurosci. Lett. 307 (2001) 29–32.
[9] K. Tabuchi, S. Tsuji, K. Fujihira, K. Oikawa, A. Hara, J. Kusakari, Outer hair cells functionally and structurally deteriorate during reperfusion, Hear. Res. 173 (2002) 153–163.
[10] T. Yamasoba, J. Schacht, F. Shoji, J.M. Miller, Attenuation of cochlear damage from noise trauma by an iron chelator, a free radical scavenger and glial cell line-derived neurotrophic factor in vitro, Brain Res. 815 (1999) 317–325.
[11] H. Murashita, K. Tabuchi, T. Hoshino, S. Tsuji, A. Hara, The effects of tempol, 3-aminobenzamide and nitric oxide synthase inhibitors on acoustic injury of the mouse cochlea, Hear. Res. 214 (2006) 1–6.
[12] T. Hoshino, K. Tabuchi, Y. Hirose, I. Uemaetomari, H. Murashita, T. Tobita, A. Hara, The non-steroidal anti-inflammatory drugs protect mouse cochlea against acoustic injury, Tohoku J. Exp. Med. 216 (2008) 53–59.
[13] Y.H. Choung, A. Taura, K. Pak, S.J. Choi, M. Masuda, A.F. Ryan, Generation of highly-reactive oxygen species is closely related to hair cell damage in rat organ of corti treated with gentamicin, Neuroscience 161 (2009) 214–226.
[14] C. Riva, E. Donadieu, J. Maqnan, J.P. Lavielle, Age-related hearing loss in CD/1 mice is associated to ROS formation and HIF target proteins up-regulation in the cochlea, Exp. Gerontol. 42 (2007) 327–336.
[15] S.L. McFadden, D.L. Ding, A.G. Reaume, D.G. Flood, R.J. Salvi, Age-related cochlear hair cell loss is enhanced in mice lacking copper/zinc superoxide dismutase, Neurobiol. Aging 20 (1999) 1–8.
[16] S.L. McFadden, D.L. Ding, R.F. Burkard, H. Jiang, A.G. Reaume, D.G. Flood, R.J. Salvi, Cu/Zn SOD deficiency potentiates hearing loss and cochlear pathology in aged 129, CD-1 mice, J. Comp. Neurol. 413 (1999) 101–112.
[17] K.K. Ohlemiller, S.L. McFadden, D.L. Ding, D.G. Flood, A.G. Reaume, E.K. Hoffman, R.W. Scott, J.S. Wright, G.V. Putcha, R.J. Salvi, Targeted deletion of the cytosolic Cu/Zn-superoxide dismutase gene (Sod1) increases susceptibility to noise-induced hearing loss, Audiol. Neurotol. 4 (1999) 237–246.
[18] D.A. Fairfield, A.C. Kanicki, M.I. Lomax, R.A. Altschuler, Induction of heat shock protein 32 (Hsp32) in the rat cochlea following hyperthermia, Hear. Res. 188 (2004) 1–11.
[19] H.J. Kim, H.S. So, J.H. Lee, J.H. Lee, C. Park, S.Y. Park, Y.H. Kim, M.J. Youn, S.J. Kim, S.Y. Chung, K.M. Lee, R. Park, Heme oxygenase-1 attenuates the cisplatin-induced apoptosis of auditory cells via down-regulation of reactive oxygen species generation, Free Radic. Biol. Med. 40 (2006) 1810–1819.
[20] H.S. So, H.J. Kim, J.H. Lee, J.H. Lee, S.Y. Park, C. Park, Y.H. Kim, J.K. Kim, K.M. Lee, K.S. Kim, S.Y. Chung, W.C. Jang, S.K. Moon, H.T. Chung, R.K. Park, Flunarizine induces Nrf2-mediated transcriptional activation of heme oxygenase-1 in protection of auditory cells from cisplatin, Cell Death Differ. 13 (2006) 1763–1775.
[21] J. Kil, C. Pierce, H. Tran, R. Gu, E.D. Lynch, Ebselen treatment reduces noise induced hearing loss via the mimicry and induction of glutathione peroxidase, Hear. Res. 226 (2007) 44–51.
[22] K. Tabuchi, M. Suzuki, A. Mizuno, A. Hara, Hearing impairment in TRPV4 knockout mice, Neurosci. Lett. 382 (2005) 304–308.
[23] K. Tabuchi, K. Pak, E. Chavez, A.F. Ryan, Role of inhibitor of apoptosis protein in gentamicin-induced cochlear hair cell damage, Neuroscience 149 (2007) 213–222.
[24] W.H. Chung, K. Pak, B. Lin, N. Webster, A.F. Ryan, A PI3K pathway mediates hair cell survival and opposes gentamicin toxicity in neonatal rat organ of Corti, J. Assoc. Res. Otolaryngol. 7 (2006) 373–382.
[25] T. Suzuki, V.P. Kelly, H. Motohashi, O. Nakajima, S. Takahashi, S. Nishimura, M. Yamamoto, Deletion of the selenocysteine tRNA gene in macrophages and liver results in compensatory gene induction of cytoprotective enzymes by Nrf2, J. Biol. Chem. 283 (2008) 2021–2030.
[26] X.Z. Liu, D. Yan, Ageing and hearing loss, J. Pathol. 211 (2007) 188–197.
[27] L. Kong, X. Zhou, J.F. McGinnis, F. Li, W. Cao, Molecular mechanisms underlying cochlear degeneration in the tubby mouse and the therapeutic effect of sulforaphane, Neurochem. Int. 54 (2008) 172–179.
[28] J. Sun, H. Hoshino, K. Takaku, O. Nakajima, A. Muto, H. Suzuki, S. Tashiro, S. Takahashi, S. Shibahara, J. Alam, M.M. Taketo, M. Yamamoto, K. Igarashi, Hemoprotein Bach1 regulates enhancer availability of hemo oxygenase-1 gene, EMBO J. 21 (2002) 5216–5224.
[29] J. Zhang, T. Ohta, A. Maruyama, T. Hosoya, K. Nishikawa, J.M. Maher, S. Shibahara, K. Itoh, M. Yamamoto, BRG1 interacts with Nrf2 to selectively mediate HO-1 induction in response to oxidative stress, Mol. Cell. Biol. 26 (2006) 7942–7952.

Ototoxicity: Mechanisms of Cochlear Impairment and its Prevention

K. Tabuchi*, B. Nishimura, M. Nakamagoe, K. Hayashi, M. Nakayama and A. Hara

Department of Otolaryngology, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tsukuba, Japan

Abstract: Aminoglycosides, cisplatin, and non-steroidal anti-inflammatory drugs (NSAIDs) are widely used pharmacological agents. There is a possibility, however, that the use of these agents may induce transient or permanent hearing loss and tinnitus as side effects. Recent animal studies have clarified mechanisms leading to the ototoxicity induced by these agents, at least in part. The permanent hearing loss caused by aminoglycosides and cisplatin is suggested to be predominantly associated with the apoptotic death of outer hair cells. Both drugs generate reactive oxygen species (ROS) in the inner ear. ROS can activate cell-death pathways such as the c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) pathways, which in turn, induce hair cell apoptosis. On the other hand, the abuse of NSAIDs may transiently cause tinnitus and mild to moderate hearing loss. NSAIDs impair the active process of the outer hair cells and affect peripheral and central auditory neurons. Conversely, recent reports clarified that NSAIDs are potential therapeutic agents against cochlear injuries. In this review, recent findings from animal studies regarding the ototoxicity induced by aminoglycosides, cisplatin, and NSAIDs are summarized. Their ototoxic mechanisms are focused on.

Keywords: Aminoglycosides, cisplatin, non-steroidal anti-inflammatory drugs (NSAIDs), ototoxicity.

INTRODUCTION

Hearing impairment can result from the use of chemotherapeutic agents, such as aminoglycoside antibiotics, cisplatin, and non-steroidal anti-inflammatory drugs (NSAIDs). Although these agents are useful and widely employed, ototoxicity, as a side effect, may restrict their uses. Basic research using experimental animals have helped to clarify, in part, the mechanisms by which these agents impair the function of the inner ear. The aim of this review is to provide an overview of recent insights regarding the ototoxicity of these three important classes of pharmaceutical.

AMINOGLYCOSIDES

Aminoglycoside antibiotics are bactericidal aminoglyosidic aminocyclitols that exhibited a therapeutic action by inhibiting bacterial protein synthesis. They bind to the bacterial 30S ribosomal subunit and inhibit the elongation of bacterial protein synthesis. They were developed mainly in 1940s to treat Gram-negative bacteria that were not responsive to the conventional antibiotics of that time, such as penicillin. Despite side effects such as nephrotoxicity and ototoxicity, aminoglycosides are still useful today to treat tuberculosis and other serious bacterial infections. The aminoglycoside superfamily comprises the compounds amikacin, dibekacin, dihydrostreptomycin, fradiomycin, gentamicin, isepamicin, kanamycin, netilmicin, streptomycin, and tobramycin. Streptomycin, the first member of aminoglycosides, was isolated from *Streptomyces griseus* between 1939 and 1944 [1]. The three most commonly prescribed aminoglycosides are gentamicin, tobramycin, and amikacin, and gentamicin is currently the most widely used aminoglycoside [2, 3]. The incidence of ototoxicity when these drugs are administered for several days reportedly lies in the 20% range [4, 5]. The incidence can increase to about 80% when administered for months, which is often necessary for the treatment of tuberculosis [6]. Recent studies have demonstrated that aminoglycoside ototoxicity begins with the disarray of hair cell stereocilia, leading to their complete disappearance, along with the degeneration and death of hair cells [7].

*Address correspondence to this author at the Department of Otolaryngology, Graduate School of Comprehensive Human Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, 305-8575, Japan; Tel: +81-298-53-3147; Fax: +81-298-53-3147;
E-mail: ktabuchi@md.tsukuba.ac.jp

MECHANISM OF AMINOGLYCOSIDE OTOTOXICITY

Reactive Oxygen Species

The generation of reactive oxygen species (ROS) is thought to be the initiating step of aminoglycoside ototoxicity. ROS are products of oxygen metabolism, which are highly reactive and potentially destructive to cell constituents, resulting in necrotic or apoptotic cell death. The formation of ROS by aminoglycosides has been observed *in vitro* in the presence of iron salt [8] as well as in cultured cells exposed to these agents [9-11]. It is considered that aminoglycosides achieve their redox capacity by binding to the transition metal iron. An *in vitro* study demonstrated that aminoglycoside antibiotics increased luminescence on employing lucigenin or luminol in cultured cells, and this luminescence was inhibited by the addition of iron chelators [11]. The redox activity of the aminoglycoside-iron complex was also revealed on the monitoring of arachidonic acid peroxidation [8]. In addition, the supplemental administration of iron exacerbated aminoglycoside ototoxicity [12].

The critical roles of ROS in hair cell death induced by aminoglycosides are supported by the observation that mice overexpressing superoxide dismutase are resistant to aminoglycoside ototoxicity [13]. Additionally, the participation of reactive nitrogen species in aminoglycoside ototoxicity has also been reported [14].

Apoptotic Hair Cell Death

The excess generation of ROS is thought to trigger pathways of apoptotic cell death. Apoptosis primarily occurs through the activation of caspases by either an extrinsic or intrinsic pathway. The extrinsic pathway begins with the activation of cell surface death receptors that are members of the tumor necrosis factor (TNF) family of receptors. Activation of these death receptors cleaves caspase-8, which, in turn, activates downstream effector caspases such as caspase-3. In contrast, the intrinsic pathway is initiated by changes in mitochondrial membrane permeability. Cytochrome c released from mitochondria forms a protein complex with Apaf 1. This, in turn, activates caspase-9 and the downstream effector caspases. The activated effector caspases then cleave critical intracellular proteins to induce the final stages of cell death [15].

The current literature suggests that the intrinsic apoptotic pathway is the major pathway of hair cell death induced by aminoglycosides in the cochlea [16-19]. Caspase-3 and -9 inhibitors but not caspase-8 inhibitors decreased auditory hair cell loss

0929-8673/11 $58.00+.00

mediated by gentamicin [19]. However, caspase-9 inhibitors did not reduce hair cell apoptosis induced by gentamicin when the apoptotic pathway was enhanced by inhibitors of the X-linked inhibitor of apoptosis protein (XIAP) [19]. In addition, caspase-8, as well as caspase-3 and caspase-9, was activated by neomycin in utricular hair cells [17]. These findings suggest that the extrinsic apoptotic pathway or cross-talk between the intrinsic and extrinsic pathways may also be involved in the aminoglycoside-induced apoptosis of hair cells.

Mitogen-activated protein kinases (MAPKs) are serine-threonine kinases that mediate intracellular signaling associated with a variety of cellular activities including cell proliferation, differentiation, survival, death, and transformation. The mammalian MAPK family consists of extracellular signal-regulated kinase (ERK), p38 MAPK, and c-Jun NH_2-terminal kinase (JNK). JNK is also known as stress-activated protein kinase (SAPK). The JNK and p38 signaling pathways are activated by various stimuli such as oxidative stress and pro-inflammatory cytokines [20]. Several reports indicated that the JNK pathway was activated during the course of the aminoglycoside-induced apoptosis of hair cells, and that aminoglycoside-induced hearing loss was alleviated by JNK inhibitors [21-23]. Another report indicated that the p38 pathway is involved in hair cell death in aminoglycoside ototoxicity [24]. In addition to the excessive administration of gentamicin, acoustic overstimulation and aging are also known to activate p38 MAPK in the cochlea [25, 26].

PREVENTION OF AMINOGLYCOSIDE OTOTOXICITY

ROS are considered to be involved in various types of cochlear injury [27]. Several chemical agents have been investigated to prevent the initial stages of lipid peroxidation and cell damage by blocking the formation of ROS or scavenging ROS once these radicals are formed [28]. These include vitamin E [29], D-methionine [30], α-lipoic acid [31], mannitol [32], EGb 761 [33], and a superoxide dismutase (SOD) mimetic [34]. Other compounds that display antioxidant properties and have recently been investigated as protective agents against aminoglycoside ototoxicity include iron chelators [32, 35] and salicylate [36].

In addition to protecting the cochlea from oxidative stress that would promote cell death, it could also be applied for cochlear protection to slow or reverse the process of apoptotic cell death. Since apoptosis participates in aminoglycoside-induced ototoxicity, trials with compounds that can block aminoglycoside-induced apoptosis have recently been a focus of studies. Caspase-3 and -9 inhibitors alleviated gentamicin ototoxicity [19]. IAPs represent a family of endogenous caspase inhibitors that share a conserved structure known as the BIR (baculovirus inhibitory repeat) domain, a zinc-binding region consisting of less than 70 amino acids [37]. A pharmacological experiment suggested that XIAPs normally act to limit hair cell death during gentamicin ototoxicity [19].

MAPKs may also be targets of therapy against aminoglycoside ototoxicity. JNK inhibitors, CEP1347 and SP600125, have been shown to reduce the aminoglycoside-induced apoptosis of hair cells [21, 23]. Estradiol, the most potent estrogen, suppressed gentamicin ototoxicity by inhibiting the activation of the JNK pathway [23]. Minocycline is known to be an inhibitor of caspases and cytochrome-c-release into the cytoplasm, and minocycline has been shown to reduce the activation of p38 MAPK and protect hair cells against gentamicin ototoxicity [24, 38].

The sphingolipid metabolites ceramide, sphingosine, sphingosine-1-phosphate (S1P), and gangliosides are known as a new class of lipid second messengers and reportedly play essential roles in the regulation of cell proliferation, survival, and death [39]. Nishimura *et al.* [40] reported that ceramide accelerated the gentamicin-induced apoptosis of outer hair cells, and, conversely, S1P inhibited hair cell apoptosis. These findings suggest that inhibition of the synthesis and/or action of ceramide as well as S1P agonists may serve for cochlear protection. In addition, protective effects of gangliosides GM1 and GM3 against gentamicin ototoxicity were also reported [40] (Fig. **1**).

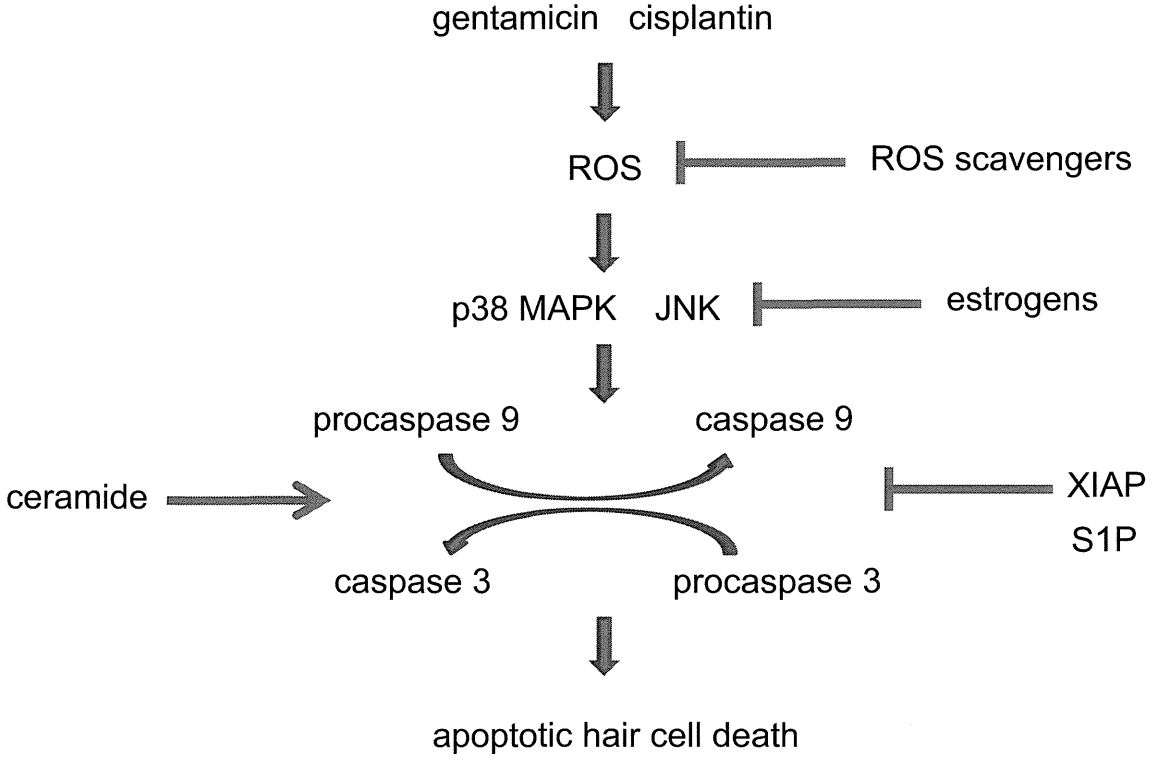

Fig. (1). A putative model of the signaling events leading to apoptosis of cochlear hair cells following exposure to aminoglycoside and cisplatin. Aminoglycoside and cisplatin induce the generation of ROS, activation of p38 MAPK and JNK, and cleavage of caspases.

In addition to the agents described above, glucocorticoids [41, 42], an enhancer of KCNQ4 (Kv7.4) potassium channels [43], and neurotrophic growth factors [44] also exert protective effects against aminoglycoside ototoxicity.

GENOTYPE AND SUSCEPTIBILITY TO AMINOGLYCOSIDES

Several mitochondrial mutations are associated with non-syndromic and syndromic hearing loss, including 1555A>G, 3243A>G, 1494C>T, and 1095T>C. The 1555A>G mutation is the most common mutation attributed to aminoglycoside-induced sensorineural hearing loss [45, 46]. Aminoglycosides exert their antibacterial effect by specifically binding to the bacterial ribosome, thus inhibiting protein synthesis or inducing the mistranslation of messenger RNAs [47]. Prezant *et al.* [48] first demonstrated that 1555A>G mutation causes the structure of the mitochondrial 12S ribosomal RNA (rRNA) to be more similar to that of bacterial rRNA, thus making the mitochondrial ribosomal decoding site more accessible to aminoglycoside antibiotics. Since then, multiple reports have confirmed this association [49].

CISPLATIN

Cisplatin is one of the most active platinum compounds in experimental tumor systems, and was introduced into clinical chemotherapy in the early 1970s. Today, it is used for the treatment of cancers of the head and neck, lung, ovary, bladder, and testis. Although the treatment of cancers with this drug is effective, serious adverse effects such as nausea, nephrotoxicity, neurotoxicity, and ototoxicity may occur during or after the course of treatment. These adverse effects often limit the dose of cisplatin. Experimental studies have demonstrated that cisplatin ototoxicity begins with damage to the first row of outer hair cells in the basal turn of the cochlea, and progresses to the other rows of outer hair cells in the organ of Corti [50]. Besides the organ of Corti, cisplatin also affects the spiral ganglion and stria vascularis. Van Ruijven *et al.* [51] demonstrated that the time sequence of damage to spiral ganglion and outer hair cells showed a similar time course. In addition, a strong correlation was reported among the damage of marginal cells of the stria vascularis, damage of hair cells, and hearing loss [52]. Those findings suggest that cisplatin injury occurs in parallel in the organ of Corti, spiral ganglion, and stria vascularis. The clinical presentation of cisplatin ototoxicity includes tinnitus and high-frequency sensorineural hearing loss, which can be permanent and progressive, and involves the lower frequencies as toxicity progresses. The incidence of ototoxicity due to cisplatin therapy varies in different clinical treatment data. For example, De Jongh *et al.* [53] suggested that 42% of patients receiving weekly cisplatin at a dose of 70-85 mg/m^2 showed signs of hearing loss. Risk factors that increase the incidence of cisplatin ototoxicity include: renal disease, larger cumulative doses, pre-existing hearing loss, younger age in children, and irradiation of the skull base [1, 54-56].

MECHANISM OF CISPLATIN OTOTOXICITY

Reactive Oxygen Species

ROS plays a crucial role in cisplatin as well as aminoglycoside ototoxicity [9]. The administration of ototoxic doses of cisplatin to experimental animals results in the depletion of glutathione and antioxidant enzymes (SOD, catalase, glutathione peroxidase, and glutathione reductase) in cochlear tissues, with a corresponding increase in malondialdehyde levels [57]. Furthermore, it is thought that reactive nitrogen species also contribute to cisplatin ototoxicity [58, 59]. Kim *et al.* [60] recently examined the roles of NADPH oxidases in cisplatin-induced ROS generation. Their immunohistochemical studies demonstrated that cisplatin induced the expression of NADPH oxidase isoforms NOX-1 and NOX-4 in HEI-OC1 auditory cells. Expression of mRNA for NOX-1, NOX-4, NOXO1, NOXA1, p47(phox), and p67(phox) was also increased. The inhibition of NADPH oxidase with diphenyleniodonium chloride or apocynin abolished ROS production and the subsequent apoptotic cell death in cisplatin-treated cells. Their data suggest that ROS were generated, in part, through the activation of NADPH oxidase in cisplatin ototoxicity [60].

Rybak *et al.* [1] suggest that the reduction of cochlear antioxidant enzymes activities may result from: (1) direct binding of cisplatin to essential sulfhydryl groups within the enzymes; (2) depletion of copper and selenium, which are essential for superoxide dismutase and glutathione peroxidase activities [61]; (3) increased ROS and organic peroxides which inactivate antioxidant enzymes [62]; and (4) depletion of glutathione and the cofactor NADPH, which are essential for glutathione peroxidase and glutathione reductase activities [63]. The depletion of antioxidant enzymes increases ROS formation in the cochlea.

Apoptotic Hair Cell Death

Recent studies have suggested that apoptosis participates not only in aminoglycoside but also cisplatin ototoxicity. As perfusion of the cochlea with a caspase-8 inhibitor was not effective in preventing either cisplatin-induced hair cell death or hearing loss, the apoptosis of cochlear hair cells induced by cisplatin does not appear to be dependent on the activation of caspase-8 [64]. Moreover, because caspase-3 and -9 are activated in cisplatin-damaged hair cells, and because the intracochlear perfusion of inhibitors of these caspases prevented apoptosis and hearing loss, it is likely that cisplatin ototoxicity is mediated by mitochondrial damage in the affected hair cells, with the sequential activation of initiator and effector caspases, resulting in apoptosis, hair cell destruction, and hearing loss [64].

PREVENTION OF CISPLATIN OTOTOXICITY

Since the formation of ROS is thought to be one of the most important factors initiating cisplatin ototoxicity, strategies to prevent this ototoxicity have involved the administration of free radical scavengers, such as amifostine [65], acetylcysteine [66], salicylates [67], and vitamin E [68]. These agents prevent the reactions of ROS with cellular proteins, lipids and DNA. Inducible NOS inhibitors and ebselen, a peroxynitrite scavenger, also protected the cochlea against cisplatin ototoxicity [57-59]. Other strategies involve the coadministration of compounds (sodium thiosulfate [22] and D-methionine [69]) which are able to induce the production of endogenous antioxidants or agents such as adenosine agonists [70] that can prevent the formation of ROS.

The protective effects of apoptosis inhibitors on cisplatin ototoxicity have also been reported. Wang *et al.* [64] reported that a caspase-3 inhibitor (z-DEVD-fmk) and caspase-9 inhibitor (z-LEHD-fmk) reduced the incidence of apoptosis, hair cell loss, and hearing threshold elevation induced by cisplatin in the guinea pig. Gene therapy with adeno-associated virus (AAV) containing a gene encoding XIAP protected the cochlea from cisplatin ototoxicity [71].

In addition to ROS scavengers and apoptosis inhibitors, dexamethasone [72, 73], flunarizine [74], a copper transporter inhibitor [75], and neurotrophins such as neurotrophin-3 (NT-3) [76] and brain-derived nerve growth factor (BDNF) [77] also exhibited protective effects against cisplatin ototoxicity.

NSAIDs

NSAIDs are usually used as painkillers, antipyretics, etc., and are some of the most commonly used drugs in daily clinical

practice. Although these agents are often useful to treat infectious or neoplastic lesions and to alleviate the patients' symptoms, their incorrect usage may cause serious problems. Those included are gastric mucosal injury, renal function impairment, allergic reactions, ototoxicity, and cardio-vascular complications [78]. Regarding ototoxicity induced by NSAIDs, tinnitus is often the first subjective symptom, and mild to moderate hearing loss, usually reversible, subsequently tends to occur. The severity of hearing loss is reportedly correlated with the plasma salicylate level [79]. Chyka *et al.* [80] stated that poison control centers in the U.S. reported 40,405 human exposures to salicylates in 2004, and that 63% of these cases were unintentional exposures.

MECHANISM OF NSAID OTOTOXICITY

Recent animal studies suggested that outer hair cells comprise one of the main sites of NSAID ototoxicity. In animal tests, a reduction in the level of otoacoustic emission (OAE), an indicator of the function of outer hair cells, was observed after the administration of high-dose salicylate [82, 82]. Intra- or extracellularly applied salicylate impaired the motility of isolated outer hair cells of the guinea pig [83]. The perilymphatic perfusion of a high concentration of salicylate decreased the CAP threshold, an indicator of the hearing level, in guinea pigs, inducing mild to moderate hearing loss [84]. High-dose NSAID medication inhibits cochlear movement which can be measured by laser interferometry [85]. On the other hand, NSAIDs did not affect the endocochlear potential (EP), an indicator of the function of the stria vascularis [86-88]. All these data obtained from animal studies suggest that high-dose NSAIDs cause the impairment of the active process, the mechano-sensory function of the outer hair cells, of the cochlea. Regarding the morphology, abnormality of the stereocilia of hair cells was observed after high-dose NSAID treatment by electron microscopic examinations [89]. NSAID ototoxicity also reportedly leads to a reduction of the OAE level in humans [90], suggesting that the same mechanism is involved in humans.

Although mild to moderate sensorineural hearing loss induced by salicylate has been attributed to impaired sound amplification by outer hair cells through its direct action on their motility, there is a disparity in salicylate concentrations between clinical and animal studies, i.e., extremely high extracellular concentrations of salicylate (from 1 to 30 mM) are required to directly induce a significant reduction of electromotility in animal studies. The concentrations are above the clinical range for humans. In contrast to the concentrations reported in animal studies, the salicylate concentrations in human plasma that induce hearing loss range from 0.1 to 1 mM [79, 91]. Wu *et al.* [91] recently reported that the clinical concentration range of salicylate caused concentration-dependent and reversible reductions in I(K,n) (KCNQ4) and subsequently depolarized outer hair cells. They suggested that this reversible I(K,n) reduction consequently reduces the driving force for the transduction current and electromotility of outer hair cells. Based on their findings, Wu *et al.* [91] proposed that this I(K,n) reduction might cause the otologic side effects of salicylate.

In addition to the dysfunction of hair cells, high-dose salicylate also affects the function of the cochlear auditory neurons [92, 93]. Salicylate inhibits cyclooxygenase (COX) that converts arachidonic acid to prostaglandin H2. N-methyl-D-aspartate (NMDA) currents were potentiated by arachidonic acid [94]. Although fast excitatory synaptic neurotransmission is predominantly mediated by α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptors in the cochlea [95, 96], Guitton *et al.* [97] suggested that the inhibition of COX is one of the key mechanisms responsible for the generation of tinnitus induced by salicylate *via* the activation of cochlear NMDA receptors. Furthermore, salicylate induces the abnormal excitability of neurons in the brainstem including the dorsal cochlear nucleus, subcortical area, and auditory cortex [98-101]. In addition, salicylate increases the mRNA expression level of the NMDA (NR2B) gene in the cochlea and midbrain [102]. Based on recent evidence from both evoked potentials and neuron-pair synchrony measures, it is unlikely that tinnitus is the expression of a set of independently firing neurons, and is more likely the result of a pathologically increased synchrony between sets of neurons [103]. Thus, in addition to the impairment of outer hair cells, changes in the excitability of auditory peripheral or central neurons may be the cause of the otological side effects of salicylate [78].

POSSIBLE APPLICATION OF NSAIDs FOR COCHLEAR PROTECTION

Although NSAIDs may cause ototoxicity in some situations, their protective effects on cochlear injuries have been also reported in animal studies. NSAIDs reportedly exhibit protective effects on the inner ear against acoustic injury [104-108] and ototoxicity induced by aminoglycosides or cisplatin [36, 67, 109]. At present, their protective effects are considered to be explained by their antioxidant properties and/or inhibition of eicosanoids [107]. Regarding subtypes of NSAIDs, Hoshino *et al.* [107] reported that inhibitors of COX-1 or lipooxygenase (LOX) but not COX-2 inhibitors protected the cochlea against acoustic injury. Based on this finding, it is assumed that it is important to consider subtypes of NSAID for cochlear protection.

CONCLUSION

Ototoxicity is an undesirable side effect that can affect large numbers of patients undergoing treatment with aminoglycoside antibiotics and cisplatin chemotherapy. Both elicit hair cell loss initiating in the basal turn of the cochlea. There are certain similarities, as well as other unexplained differences, in the ototoxic effects of aminoglycoside antibiotics and cisplatin. ROS are reportedly predominant initiating causes of these injuries. Hair cell loss occurs, at least in part, through apoptotic processes, and ROS are considered to cause apoptotic hair cell death *via* MAPK pathways. Recent experimental research has provided evidence that aminoglycoside and cisplatin ototoxicity can be reduced by the use of chemical agents that block the production of ROS, scavenge ROS, or inhibit the apoptotic pathways. On the other hand, NSAID ototoxicity is observed predominantly on the inappropriate use of these agents. NSAID abuse generally causes transient hearing loss and tinnitus. Although the abuse of NSAIDs may induce ototoxicity, their protective actions against various cochlear injuries have recently attracted the attention of researchers. Additional research is essential to clarify the generation mechanisms underlying the ototoxicity of aminoglycosides, cisplatin, and NSAIDs in order to develop more selective and specific strategies to protect the cochlea.

ACKNOWLEDGEMENT

This work was supported by a Grant-in-aid for Scientific Research ((C) 20591969) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

REFERENCES

[1] Rybak, .L.P.; Ramkumar, V. Ototoxicity. *Kindey Int.*, **2007**, *72*, 931-935.
[2] Santucci, R.A.; Krieger, J.N. Gentamicin for the practicing urologist: review of efficacy, single dailydosing and "switch" therapy. *J. Urol.*, **2000**, *163*, 1076-1084.
[3] Guthrie, O.W. Aminoglycoside induced ototoxicity. *Toxicology*, **2008**, *249*, 91-96.
[4] Moore, R.D.; Smith, C.R.; Lietman, P.S. Risk factors for the development of auditory toxicity in patients receiving aminoglycosides. *J. Infect. Dis.*, **1984**, *149*, 23-30.
[5] Lerner, S.A.; Schmitt, B.A.; Seligsohn, R.; Matz, G.J. Comparative study of ototoxicity and nephrotoxicity in patients randomly assigned to treatment with amikacin or gentamicin. *Am. J. Med.*, **1986**, *80*, 98-104.

[6] Brouet, G.; Marche, J.; Chevallier, J.; Liot, F.; Le Meur, G.; Bergogne. Experimental and clinical studies on kanamycin in tuberculous infection. *Rev. Tuberc. Pneumol. (Paris)*, **1959**, *23*, 949-988.

[7] Waguespack, J.R.; Ricci, A.J. Aminoglycoside ototoxicity: permeant drugs cause permanent hair cell loss. *J. Physiol.*, **2005**, *567*, 505-521.

[8] Priuska, E.; Schacht, J. Formation of free radicals by gentamicin and iron and evidence for an iron/gentamicin complex. *Biochem. Pharmacol.*, **1995**, *50*, 1749-1752.

[9] Clerici, W.J.; Hensley, K.; DiMartino, D.L.; Buttrfield, D.A. Direct detection of ototoxicant-induced reactive oxygen species generation in cochlear explants. *Hear. Res.*, **1996**, *98*, 116-124.

[10] Hirose, K.; Hockenbery, D.M.; Rubel, E.W. Reactive oxygen species in chick hair cells after gentamicin exposure *in vitro*. *Hear. Res.*, **1997**, *104*, 1-14.

[11] Sha, S.H.; Schacht, J. Stimulation of free radical formation by aminoglycoside antibiotics. *Hear. Res.*, **1999**, *128*, 112-118.

[12] Canlon, B.J.; Smith, D.W. Supplemental iron exacerbates animoglycoside ototoxicity *in vitro*. *Hear. Res.*, **1998**, *115*, 1-5.

[13] Sha, S.H.; Zajic, G.; Epstein, C.J.; Schacht, J. Overexpression of copper/zinc-superoxide dismutase protects from kanamycin-induced hearing loss. *Audiol. Neurootl.*, **2001**, *6*, 117-123.

[14] Takumida, M.; Anniko, M. Nitric oxide in guinea pig vestibular sensory cells following gentamicin exposure *in vitro*. *Acta Otolaryngol.*, **2001**, *121*, 346-350.

[15] Van De Water, T.R.; Lallemend, F.; Eshraghi, A.A.; Ahsan, S.; He, J.; Guzman, J.; Polak, M.; Malgrange, B.; Lefebvre, P.P.; Staecker, H.; Balkany, T.J. Caspases, the enemy within, and their role in oxidative stress-induced apoptosis of inner ear sensory cells. *Otol. Neurootol.*, **2004**, *25*, 627-632.

[16] Bodmer, D.; Brors, D.; Pak, K.; Bodmer, M.; Ryan, A.F. Gentamicin-induced hair cell death is not dependent on the apoptosis receptor Fas. *Laryngoscope*, **2003**, *113*, 452-455.

[17] Cunningham, L.L.; Cherg, A.G.; Rubel, E.W. Caspase activation in hair cells of the mouse utricle exposed to neomycin. *J. Neurosci.*, **2002**, *22*, 8532-8540.

[18] Dehne, N.; Rauen, U.; de Groot, H.; Lautermann, J. Involvement of the mitochondrial permeability transition in gentamicin ototoxicity. *Hear. Res.*, **2002**, *169*, 47-55.

[19] Tabuchi, K.; Pak, K.; Chavez, E.; Ryan, A.F. Role of inhibitor of apoptosis protein in gentamicin-induced cochlear hair cell damage. *Neuroscience*, **2007**, *149*, 213-222.

[20] Kim, E.K.; Choi, E-J. Pathological roles of MAPK signaling pathways in human diseases. *Biochim. Biophys. Acta*, **2009**, *1802*, 396-405.

[21] Ylikoski, J.; Xing-Qun, L.; Virkkala, J.; Pirvola, U. Blockade of c-Jun N-terminal kinase pathway attenuates gentamicin-induced cochlear and vestibular hair cell death. *Hear. Res.*, **2002**, *166*, 33-43.

[22] Wang, J. Llovd Faulconbridge, R.V.; Fetoni, A.; Guitton, M.J.; Pujol, R.; Puel, J.L. Local application of sodium thiosulfate prevents cisplatin-induced hearing loss in the guinea pig. *Neuropharmacology,* **2003**, *45*, 380-393.

[23] Nakamagoe, M.; Tabuchi, K.; Uemaetomari, I.; Nishimura, B.; Hara, A. Estradiol protects the cochlea against gentamicin ototoxicity through inhibition of the JNK pathway. *Hear. Res.*, **2010**, *261*, 67-74.

[24] Wei, X.; Zhao, L.; Liu, J.; Dodel, R.C.; Farlow, M.R.; Du, Y. Minocycline prevents gentamicin-induced ototxicity by inhibiting p38 MAP kinase phosphorylation and caspase 3 activation. *Neuroscience*, **2005**, *131*, 513-521.

[25] Sha, S.H.; Chen, F.Q.; Schacht, J. Activation of cell death pathways in the inner ear of the aging CBA/J mouse. *Hear. Res.*, **2009**, *254*, 92-99.

[26] Tabuchi, K.; Oikawa, K.; Hoshino, T.; Nishimura, B.; Hayashi, K.; Yanagawa, T.; Warabi, E.; Ishii, T.; Tanaka, S.; Hara, A. Cochlear protection from acoustic injury by inhibitors of p38 mitogen-activated protein kinase and sequestosome 1 stress protein. *Neuroscience*, **2010**, *166*, 665-670.

[27] Tabuchi, K.; Nishimura, B.; Tanaka, S.; Hayashi, K.; Hirose, Y.; Hara, A. Ischemia-reperfusion injury of the cochlea: pharmacological strategies for cochlear protection and implications of glutamate and reactive oxygen species. *Curr. Neuropharmacol.*, **2010**, *8*, 128-134.

[28] Murashita, H.; Tabuchi, K.; Hara, A. Ototoxicity: cause of hair cell death. In Recent advances in auditory neuroscience. Eds. Tabuchi K, Hara A. Research signpost. Kerala. **2007**. Pp. 19-26.

[29] Fetoni, A.R.; Sergi, B.; Scarano, E.; Paludetti, G.; Ferraresi, A.; Troiani, D. Protective effects of alpha-tocopherol against gentamicin-induced Oto-vestibulo toxicity: an experimental study. *Acta Otolaryngol.*, **2003**, *123*, 192-197.

[30] Sha, S.H.; Schacht, J. Antioxidants attenuate gentamicin-induced free radical formation *in vitro* and ototoxicity *in vivo*: D-methionine is a potential protectant. *Hear. Res.*, **2000**, *142*, 34-40.

[31] Canlon, B.J.; Aran, J.M.; Erre, J.P.; Smith, D.W. Attenuation of aminoglycoside-induced cochlear damage with the metabolic antioxidant alpha-lipoic acid. *Hear. Res.*, **1999**, *128*, 40-44.

[32] Song, B.B.; Sha, S.H.; Schacht, J. Iron chlators protects from aminoglycoside-induced cochleo- and vestibule-toxicity. *Free Radic. Biol. Med.*, **1998**, *25*, 189-195.

[33] Yang, T.H.; Young, Y.H.; Liu, S.H. EGb 761 (Ginkgo biloba) protects cochlear hair cells against hair cells against ototoxicity induced by gentamicin *via* reducing reactive oxygen species and nitric oxide-related apoptosis. *J. Nutr. Biochem.*, **2010**, Epub ahead of print.

[34] McFadden,S.L.; Ding, D.; Salvemini, D.; Salvi, R.J. M40403, a superoxide dismutase mimetic, protects cochlear hair cells from gentamicin, but not cisplatin toxicity. *Txicol. Appl. Pharmacol.*, **2003**, *186*, 46-54.

[35] Song, B.B.; Schacht, J. Variable efficacy of radical scavengers and iron chelators to attenuate gentamicin ototoxicity in guinea pig in viro. *Hear. Res.*, **1996**, *94*, 87-93.

[36] Sha, S.H.; Schacht, J. Salicylate attenuates gentamicin-induced ototoxicity. *Lab. Invest.*, **1999**, *79*, 807-813.

[37] Schimmer, A.D. Inhibitor of apoptosis protein: translating basic knowledge into clinical practice. *Cancer Res.*, **2004**, *64*, 7183-7190.

[38] Corbacella, E.; Lanzoni, I.; Ding, D.; Previati, M.; Salvi, R. Minocycline attenuates gentamicin induced hair cell loss in neonatal cochlear cultures. *Hear. Res.*, **2004**, *197*, 11-18.

[39] Maceyka, M.; Payne, S.G.; Milstien, S.; Spiegel, S. Sphingosine kinase, sphingosine-1-phosphate, and apoptosis. *Biochim. Biophys. Acta*, **2002**, *1585*, 193-201.

[40] Nishimura, B.; Tabuchi, K.; Nakamagoe, M.; Hara, A. The influences of sphingolipid metabolites on gentamicin-induced hair cell loss of the rat cochlea. *Neurosci. Lett.*, **2010**, *485*, 1-5.

[41] Himeno, C.; Komeda, M.; Izumikawa, M.; Takemura, K.; Yagi, M.; Weiping, Y.; Doi, T.; Kuriyama, H.; Miller, J.M.; Yamashita, T. Intra-cochlear administration of dexamethasone attenuates aminoglycoside ototoxicity in the guinea pig. *Hear. Res.*, **2002**, *167*, 61-70.

[42] Tabuchi, K.; Nakamagoe, M.; Nishimura, B.; Hayashi, K.; Nakayama, M.; Hara, A. Protective effects of corticosteroids and neurosteroids on cochlear injury. *Med. Chem.*, **2011**, *7*, 140-144.

[43] Leitner, M.G.; Halaszovich, C.R.; Oliver, D. Aminoglycoside inhibit KCNQ4 channels in cochlear outer hair cells *via* depletion of phosphatidylinositol(4,5)bisphosphate. *Mol. Pharmacol.*, **2011**, *79*, 51-60.

[44] Kawamoto, K.; Yagi, M.; Stover, T.; Kanzaki, S.; Raphael, Y. Hearing and hair cells are protected by adenoviral gene therapy with TGF-beta1 and GDNF. *Mol. Ther.*, **2003**, *7*, 484-492.

[45] Zhao, H.; Li, R.; Wang, Q.; Deng, J.H.; Han, D.; Bai, Y.; Young, W.Y.; Guan, M.X. Maternally inherited aminoglycoside-induced and non-syndromic deafness is associated with the novel C1494T mutation in the mitochondrial 12S rRNA gene in a large Chinese family. *Am. J. Human Genet.*, **2004**, *74*, 139-152.

[46] Rydzanicz, M.; Wrobel, M.; Pollak, A.; Gawecki, W.; Brauze, D.; Kostrzewska-Poczekaj, M.; Wojsyk-Banaszak, I.; Lechowicz, U.; Mueller-Malesinska, M.; Oldak, M.; Pioski, R.; Skarzynski, H.; Szyfter, K. Mutation analysis of mitochondrial 12S rRNA gene in Polish patients with non-syndromic and aminoglycoside-induced hearing loss. *Biochem. Biophys. Res. Commun.*, **2010**, *395*, 116-121.

[47] Moazed, D.; Noller, H. Transfer-RNA shields specific nucleotides in 16s ribosomal-RNA from attack by chemical probes. *Cell*, **1986**, *35*, 433-436.

[48] Prezant, T.; Agapian, J.; Bohlman, M.; Bu, S.; Oztas, S.; Qiu, W.; Arnos, K.; Cortopassi, G.; Jaber, L.; Rotter, J.; Shohat, M.; Fischelghodsian, N. Mitochondrial ribosomal-RNA mutation associated with both antibiotic-induced and non-syndromic deafness. *Nat. Genet.*, **1993**, *4*, 289-294.

[49] Hobbie, S.; Bruell, C.; Akshay, S.; Kalapala, S.; Shcherbakov, D.; Bottger, E. Mitochondrial deafness alleles confer misreading of the genetic code. *Proc. Natl. Acad. Sci. USA.*, **2008**, *105*, 3244-3249.

[50] Cardinaal, R.M.; de Groot, J.C.; Huizing, E.H.; Veldman, J.E.; Smoorenburg, G.F. Cisplatin-induced ototoxicity: morphological evidence of spontaneous outer hair cell recovery in albino guinea pigs? *Hear. Res.*, **2000**, *144*,147-156.

[51] Van Ruijven, M.W.; de Groot ,J.C.; Klis, S.F.; Smoorenburg, G.F. The cochlear targets of cisplatin: an electrophysiological and morphological time-sequence study. *Hear. Res.*, **2005**, *205*, 241-248.

[52] Meech, R.P.; Cambell, K.C.; Hughes, L.F.; Rybak, L.P. A semiquantitative analysis of the effects of cisplatin on the rat stria vascularis. *Hear. Res.*, **1998**, *124*, 44-59.

[53] De Jongh, F.E.; van Veen, R.N.; Veltman, S.J.; de Wit, R.; van der Burg, M.E.; van den Bent, M.J.; Plating, A.S.; Gravelad, W.J.; Stoter, G. Weekly high-dose cisplatin is a feasible treatment option: analysis on prognostic factors for toxicity in 400 patients. *Br. J. Cancer*, **2003**, *88*, 1199-1206.

[54] Li, Y.; womer, R.B.; Silber, J.H. Predicting cisplatin ototoxicity in children: the influence of age and the cumulative dose. *Eur. J. Cancer*, **2004**, *40*, 2445-2451.

[55] Knight. K.R.G.; Kraemer, D.F.; Neuwelt, E.A. Ototoxicity in children receiving platinum chemotherapy: understanding a commonly occurring toxicity that may influence academic and social development. *J. Clin. Oncol.*, **2005**, *23*, 8588-8896.

[56] Chen, W.C.; Jackson, A.; Budnick, A.S.; Pfister Din, G.; Kraus, D.H.; Hunt, M.A.; Stambuk, H.; Levegrum, S.; Wolden, S.L. Sensorineural hearing loss in combined modality treatment of nasopharyngeal carcinoma. *Cancer*, **2006**, *106*, 820-829.

[57] Rybak, L.P.; Husain, K.; Morris, C.; Whitworth, C.; Somani, S. Effect of protective agents against cisplatin ototoxicity. *Am. J. Otol.*, **2000**, *149*, 189-198.

[58] Kelly, T.C.; Whitworth, C.A.; Husain, K.; Rybak, L.P. Aminoguanidine reduces cisplatin ototoxicity. *Hear. Res.*, **2003**, *186*, 10-16.

[59] Watanabe, K.; Hess, A.; Michel, O.; Yagi, T. Nitric oxide synthase inhibitor reduces the apoptotic change in the cisplatin-treated cochlea of guinea pigs. *Anticancer Drugs*, **2000**, *11*, 731-735.