activation of the apoptotic pathways. In addition, we also clarified the caspase 3-dependency of SC death following the silencing of $p27^{kip1}$ expression by examining explants that were cultured in medium supplemented with 100 μM Caspase-3 inhibitor III (Calbiochem, San Diego, CA) after a mixture of sh-p27a and pEGFPN-1 had been introduced. The numbers of EGFP+ cells after 2–7 days of culturing were counted ($n=5$ for each time point).

To examine the trans-differentiation of SCs into hair cells after silencing the $p27^{kip1}$ expression, myosin VIIa was labeled immunohistochemically in explants into which a mixture of sh-p27a and pEGFPN-1 had been introduced 5 or 7 days earlier ($n=5$ for each condition). Results were used to examine the trans-differentiation of SCs into hair cells after silencing the $p27^{kip1}$ expression.

Statistical analysis

The effects of silencing $p27^{kip1}$ expression and inhibiting caspase 3 on the survival of EGFP+ cells in auditory epithelia were examined using a two-way factorial analysis of variance (ANOVA). When an interaction was determined to be significant, pair-wise comparisons were analyzed using the Tukey–Kramer test for multiple comparisons. A one-way factorial ANOVA with the Tukey–Kramer test was used for analysis of differences for the number of EGFP+, BrdU+ cells among cultured periods in cochlear explants that were introduced a mixture of pEGFPN-1 and sh-p27a. The data was presented as the means ± standard deviations.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mcn.2009.08.011.

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The Laryngoscope

Hepatocyte Growth Factor Protects Auditory Hair Cells From Aminoglycosides

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Objectives/Hypothesis: To examine the effect of hepatocyte growth factor (HGF) for protection of auditory hair cells against aminoglycosides and its molecular mechanisms.

Study Design: Experimental study.

Methods: We quantitatively assessed protective effects of HGF on mouse cochlear hair cells against neomycin toxicity using explant culture systems. To understand mechanisms of hair cell protection by HGF, we examined the expression of c-Met, HGF receptor, and 4-hydroxynonenal (a lipid peroxidation marker) in the cochlea by means of immunohistochemistry and Western blotting.

Results: The application of HGF to cochlear explant cultures significantly reduced the hair cell loss induced by neomycin. Immunohistochemistry showed c-Met expression in normal auditory hair cells, and its increase in response to neomycin-induced damage. Immunostaining for 4-hydroxynonenal suggested that HGF acted by attenuating the lipid peroxidation of auditory epithelia induced by neomycin.

Conclusions: These findings demonstrate that a functional HGF/c-Met coupling is present in the cochlea, and HGF application exerts protective effects on hair cells, indicating the potential of HGF as a therapeutic agent for sensorineural hearing loss.

Key Words: Cochlea, protection, c-Met, growth factor, hearing loss, 4-hydroxynonenal.

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INTRODUCTION

Hepatocyte growth factor (HGF), which was initially identified and purified as a multifunctional protein in hepatocytes,[1] exerts its activities, such as mitogenic, morphogenic, angiogenic, and antiapoptotic activities, in various types of cells,[2,3] including neuronal cells. HGF, and its receptor c-Met, are expressed in various regions of the brain and peripheral neurons.[2–4] A functional coupling between HGF and c-Met has been reported to play important roles in the development and the maintenance of the nervous system.[5,6] Previous studies have revealed that HGF gene transfer with hemagglutinating virus of Japan envelope (HVJ-E) prevents postischemic delayed neuronal death in the hippocampus.[2,7] Kitamura et al. also delivered the HGF gene to the injured spinal cords using herpes simplex virus-1.[8] Altogether, these studies suggest that HGF functions as a neurotrophic factor to maintain the physiological structure and function of the nervous system.

Several studies indicated a therapeutic potential of HGF for inner ear disorders. Previously, Oshima et al. reported that HGF gene delivery into the cerebrospinal fluid with HVJ-E vector promotes functional recovery after aminoglycoside-induced hearing loss.[9] Recently, we have demonstrated that local application of recombinant human HGF protein into the cochlea using gelatin hydrogels,[10] which enables sustained delivery of growth factors, rescues auditory hair cells from noise-induced damage.

However, actual mechanism of HGF action to the hair cells is not yet clearly described. The present study aimed to explore the molecular mechanism of HGF effect and examine the potential of HGF. We used cochlear explant culture systems and tested the capability of HGF for protection of auditory hair cells against aminoglycoside toxicity. In addition, to explore the HGF signaling mechanism on the auditory epithelium, we examined the expression of the c-Met receptor and monitored the changes in lipid oxidation in auditory epithelia damaged by neomycin.

MATERIALS AND METHODS

Animals

ICR mice (Japan SLC, Hamamatsu, Japan) were used in this study. The Animal Research Committee, Graduate School

of Medicine, Kyoto University, approved all experimental protocols. Animal care was under the supervision of the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University. All experimental procedures were performed in accordance with the *NIH Guide for the Care and Use of Laboratory Animals.*

Cochlear Explant Culture

On postnatal day 3 (P3), ICR mice were deeply anesthetized with sevoflurane and decapitated. The temporal bones were dissected, and the cochleae were freed from surrounding tissues in 0.01 M phosphate-buffered saline (PBS) at pH 7.4. After removal of the cochlear lateral wall, auditory epithelia were dissected from the cochlear modiolus. The tissue pieces were then attached to glass mesh inserts (Falcon, Billerica, MA) and cultured initially in serum-free minimum essential medium (Invitrogen, Eugene, OR) supplemented with 3 g/L glucose and penicillin G for 24 hours. Cultures were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Because hair cells in the apex are resistant to aminoglycosides,[11,12] basal turns of the cochlea were used in this study.

Neomycin Application, Cell Survival, and HGF Protection Assay

The explants were then transferred to the medium containing neomycin sulfate (Sigma-Aldrich, St. Louis, MO) at concentrations of 0, 0.1, 0.3, 1, or 3 mM, with six to 10 cochleae incubated at each concentration. For HGF protection assay, we examined effects of 20 ng/mL recombinant human HGF (rhHGF, Sigma-Aldrich) with the same neomycin concentrations. We also used 1 mM neomycin media supplemented with recombinant human HGF at concentrations of 0, 4, 20, or 100 ng/mL to estimate optimal HGF concentration.

Cultures were maintained for 24 hours and at the end of the culture period, samples were fixed for 15 minutes in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Specimens were then rinsed with PBS, incubated in 1% bovine serum albumin (BSA) with 0.2% Triton X-100 for 30 minutes and immersed in Alexa 488-labeled phalloidin (1:200; Invitrogen) for 30 minutes. Samples were examined with a Leica TCS-SP2 confocal microscope (Leica Microsystems Inc., Wetzlar, Germany). To quantify hair cell loss in the cochlea after various treatments, inner hair cells (IHCs) and outer hair cells (OHCs) in the auditory epithelia were counted over a 100-μm longitudinal distance from two separate regions in the basal turn of each culture (totaling 200 μm).

c-Met Expression in the Cochlea

Temporal bones of P3 ICR mice (n = 4) were collected under deep anesthesia. Cochleae were perfused with saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer and immersed in the same fixative at room temperature for 2 hours. After ethylenediaminetetraacetic acid decalcification, the specimens were embedded in optimum cutting temperature compound (Tissue Tek, Miles Inc., Elkhart, IN) and sectioned at 15 μm using a cryostat. Midmodiolar sections were provided for immunohistochemistry. Sections were briefly refixed with 4% paraformaldehyde and blocked with 1% BSA with 0.2% Triton X-100 for 30 minutes. Primary antibodies were rabbit polyclonal anti-c-Met (1:100, Santa Cruz Biotechnology, Santa Cruz, CA) and anti-myosin VIIa (1:500, Proteus Bioscience Inc., Ramona, CA). Alexa-Fluor 488-conjugated goat anti-rabbit IgG (1:200; Invitrogen) were used as the secondary antibody, and Zenon Alexa Fluor 555 rabbit IgG labeling kit (Invitrogen) was used for anti-myosin VIIa staining. Specimens were then incubated in PBS containing 2 mg/mL DAPI (Invitrogen) for nuclear staining.

Alteration of c-Met Levels in Cochlear Explants

We employed Western blotting to estimate alterations of c-Met expression in cochlea explants following neomycin application. Cochlear explants were cultured without glass mesh, using medium containing neomycin sulfate at concentrations of 0, 0.3, 1, or 3 mM for 24 hours. Ten cochleae were used for each condition. Explants were then quickly collected and homogenated in ice-cold radio immunoprecipitation assay buffer with protease inhibitor cocktail (Nakalai Tesque, Kyoto, Japan). Five micrograms of each extract was separated on a 4% to 15% Tris-HCL gradient polyacrylamide gel (Bio-Rad, Hercules, CA) and then transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA). Membranes were blocked for 40 minutes with 5% BSA in Tween-TBS (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Tween 20) and stained overnight with anti-c-Met antibody or antiactin antibody (Sigma) at 4°C. After three washes the membranes were incubated in a 1:20000 dilution of horseradish peroxidase-conjugated secondary antibody for 1 hour and developed using the ECL plus Western blotting detection system (Amersham Pharmacia Biotech, Uppsala, Sweden). Experiments were repeated three times and pictures were processed with Adobe Photoshop CS (Adobe Systems, San Jose, CA), and band intensity was measured using ImageJ software (http://rsb.info.nih.gov/ij/; National Institutes of Health, Bethesda, MD).

Lipid Peroxidation Assay

Lipid peroxidation was assessed in cultures treated with neomycin, in the presence or absence of HGF, by measuring expression of 4-hydroxynonenal (HNE) immunohistochemically.[13] Explants were labeled with mouse anti-HNE monoclonal antibody (1:8; Oxis Research, Portland, OR) and Alexa-Fluor 568 goat anti-mouse IgG (1:200; Invitrogen) as the primary and secondary antibodies. Specimens were then counterstained with Alexa 488 phalloidin and examined with a fluorescence microscope. All images were taken with the same exposure and shutter speed. Red fluorescence intensity was measured using ImageJ software.

Statistical Analysis

Overall effects of HGF on hair cell numbers were analyzed by 2-way factorial analysis of variance (ANOVA) using the Statcel2 application (OMS Publishing, Saitama, Japan), with P values below .05 considered statistically significant. For interactions that were significant, multiple paired comparisons were analyzed using the Tukey-Kramer test. Measurements of HNE staining intensity were analyzed with 1-way ANOVA with the Tukey-Kramer test.

RESULTS

Dose-Dependent Hair Cell Loss by Neomycin

First, we tested a dose-response relationship between neomycin concentrations and hair cell counts. The addition of 0-3 mM neomycin for 24 hours significantly reduced hair cell numbers in both IHC and OHC regions (Fig. 1A, C, E, G). The addition of 1 mM of neomycin destroyed approximately 73% of the IHCs and 64% of the OHCs (Fig. 1E, I). The hair cell density

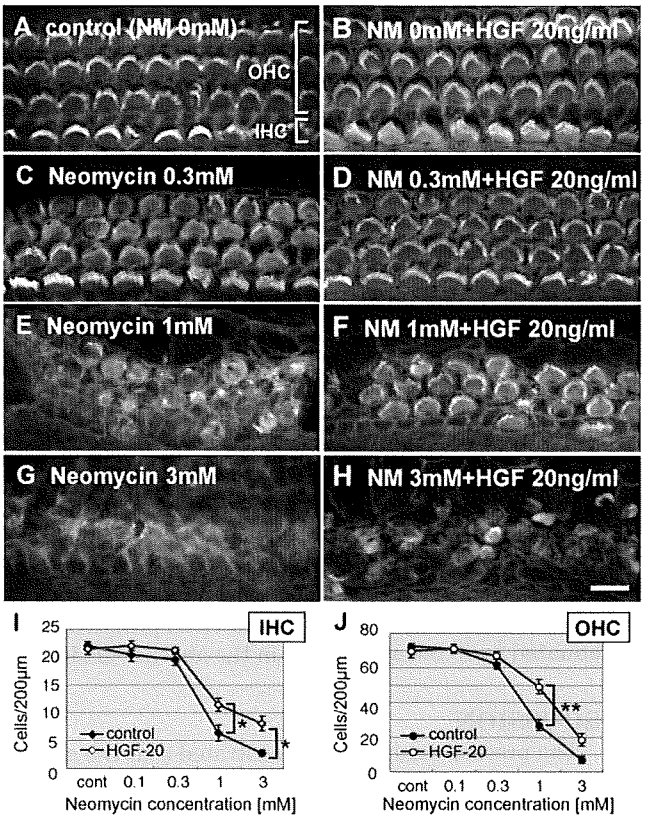

Fig. 1. Dose-dependent auditory hair cell loss by neomycin and its protection by hepatocyte growth factor (HGF). Cochleae were cultured for 24 hours in various concentrations of neomycin with or without HGF. (A–H). Photomicrographs of phalloidin-labeled cochlear cultures. Outer hair cell (OHC) consists of three rows of triangular-shaped cells that have phalloidin-positive, inverted v-shaped stereocilia bundles on top of them. Inner hair cells (IHC) are oval-shaped cells that have flat, u-shaped stereocilia bundles. Bar = 10 μm. (A). A control showing normal arrangement of IHCs and OHCs. (C, E, G). Examples of cochlear cultures treated with neomycin at a concentration of 0.3 mM (C), 1 mM (E), and 3 mM (G). (B, D, F, H). Examples of cochlear cultures treated with 20 ng/mL HGF and neomycin at a concentration of 0 mM (B), 0.3 mM (D), 1 mM (F), and 3 mM (H). (I, J). Hair cell count following neomycin and HGF-supplemented cultures. Filled symbols represent counts from control cultures without HGF and open symbols from HGF- supplemented cultures. In HGF-free cultures, the hair cell density decreased systematically as the neomycin concentration increased. HGF significantly attenuated the loss of IHC ($P < .05$) and OHC ($P < .001$) in neomycin-damaged cochleae (2-way analysis of variance). Post hoc analyses with Tukey-Kramer tests for multiple pairwise comparisons showed that IHC loss for HGF plus neomycin was significantly less ($P < .05$) than for neomycin alone at neomycin concentrations of 1 and 3 mM, and also enhanced the survival of OHCs at 1 mM neomycin ($P < .01$). *$P < .05$. **$P < .01$. Bars in (I) and (J) represent standard errors.

decreased as the neomycin dose increased until virtually all hair cells were absent from the auditory epithelium cultured in 3 mM neomycin.

Dose-Response Effects of HGF Against Neomycin

To assess whether HGF has protective effects against neomycin damage, we examined the power of HGF on a quantitative assessment of dose-response relationship of the hair cells to various concentrations of neomycin. We administered 20 ng/mL HGF to 0, 0.1, 0.3, 1, or 3 mM neomycin cultures (Fig. 1B, D, F, H). The addition of 20 ng/mL HGF markedly enhanced IHC and OHC survival. HGF even promoted the survival of a substantial number of hair cells at the highest neomycin dose, 3 mM (Fig. 1G vs. H). Two-way ANOVA analyses showed that HGF has a significant effect on both remaining IHC and OHC numbers ($P = .000097$ and $P = .0000011$, respectively). Tukey-Kramer tests for multiple pairwise comparisons showed that IHC losses for HGF at 1 or 3 mM neomycin was significantly less ($P < .05$) than those for neomycin alone conditions (Fig. 1I). Twenty ng/mL HGF also significantly ($P < .01$) enhanced the survival of OHCs at 1 mM neomycin condition (Fig. 1J). These data demonstrated that HGF exerts significant protective effects against neomycin-induced hair cell damage.

Optimal Concentration of HGF for Protection

We then administered 4 to 100 ng/mL HGF to these cultures (Fig. 2). Based on the above dose-response experiment, 1 mM neomycin was used. When HGF was administered alone to control cultures at 4 to 100 ng/mL, the number of hair cells present in the samples was comparable to untreated controls indicating that HGF alone had no negative or mitogenic effects at these concentrations (Fig. 1B). In neomycin-

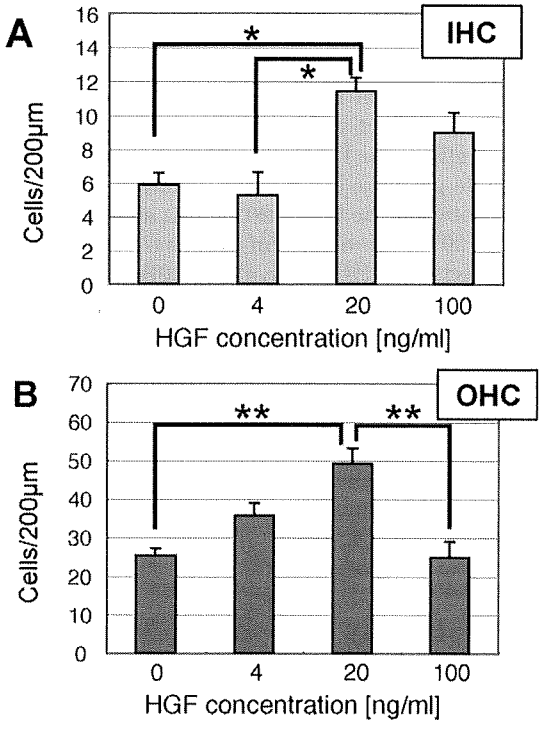

Fig. 2. Effects of different concentrations of hepatocyte growth factor (HGF) on the survival of cochlear hair cells. Cochleae were incubated with different concentrations of HGF in the presence of 1 mM neomycin. (A) Inner hair cell (IHC) and (B) outer hair cell (OHC) counts in HGF-treated cultures. HGF significantly increased hair cell survival at concentrations up to 20 ng/mL. *$P < .05$. **$P < .01$. Bars represent standard errors.

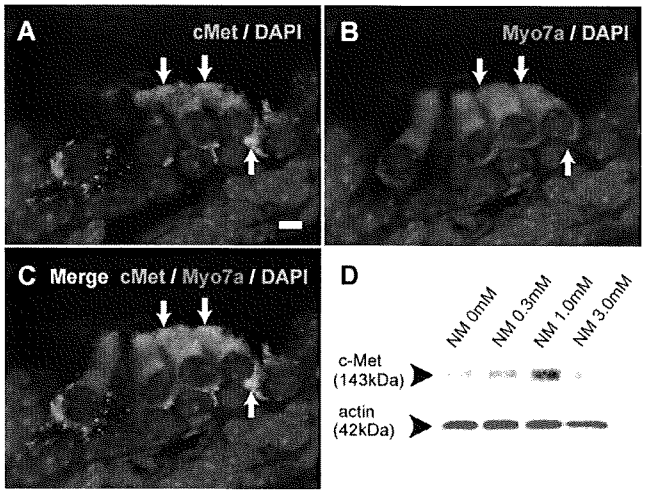

Fig. 3. c-Met expression in the organ of Corti increases after neomycin insult. (A, B, C). c-Met localization (green) in a normal postnatal day 3 cochlea. Specimen was counterstained with antimyosin VIIa (red) and DAPI (blue). Arrows indicate punctuate c-Met staining in the hair cells. Bar = 10 μm. (D). Induction of c-Met expression in the neomycin-injured cochlea. Western blotting analyses showed a remarkable increase of c-Met expression, peaking when neomycin was applied at 1.0 mM. Relative band intensities were 1.00, 1.12 $\pm$ 0.26, 1.94 $\pm$ 0.48, and 1.17 $\pm$ 0.35 for 0, 0.3, 1.0, and 3.0 mM neomycin (n = 10 in each condition).

supplemented cultures, adding 4 ng/mL of HGF had little effect on IHC survival. In comparison, HGF treatment at 20 ng/mL elicited 64% increase of remaining cell counts. However, at concentration of 100 ng/mL HGF, no further increases in the number of IHCs were seen, indicating that HGF effects saturated at 20 ng/mL concentration. A 1-way ANOVA revealed significant treatment effects for HGF ($P = .018$). Tukey-Kramer tests for multiple pairwise comparisons showed significant differences between the 0 and 20 ng/mL neomycin ($P < .05$) and 4 and 20 ng/mL neomycin groups ($P < .05$). HGF had similar effects on OHC survival (Fig. 2B). HGF treatment at 20 ng/mL elicited almost a two-fold increase of remaining cell counts. A 1-way ANOVA revealed significant effects for HGF ($P = .000049$). Tukey-Kramer tests for multiple pairwise comparisons showed significant differences between the 0 and 20 ng/mL neomycin ($P < .01$) and 20 and 100 ng/mL neomycin groups ($P < .01$). Based on these findings, the optimal concentration of HGF to 1 mM neomycin damage was estimated as 20 ng/mL for both IHCs and OHCs.

c-Met Localization in the Auditory Epithelia

To reveal whether cochlear protection mechanism of HGF is mediated by HGF/c-Met paracrine coupling, we first examined c-Met expression in the cochlea using immunohistochemistry (Fig. 3A, B, C). The expression of c-Met was detected punctate in both IHCs and OHCs, most densely at the apical region of the OHCs and the nerve ending (basal) area of IHCs and OHCs. In contrast, surrounding supporting cells expressed minimal amount of c-Met.

c-Met Expression Increases After Neomycin Insult

We then tested whether neomycin damage could increase c-Met expression in the hair cells. We examined c-Met expression in cochlear explant cultures after 24 hours of 0 to 3 mM neomycin application. Western blotting revealed that c-Met expression peaked when neomycin was applied at 1.0 mM (Fig. 3D). Relative band intensity was 1.94 $\pm$ 0.48 compared to that of 0 mM neomycin. At 3.0 mM neomycin, band intensity decreases, corresponding to fewer hair cells remaining in the auditory epithelia at this neomycin concentration (Fig. 1G).

Antioxidant Effects of HGF

We also investigated the antioxidant effect of HGF, as another mechanism by which its protective effect might function. HNE expression in explant cultures increased in the presence of neomycin (Fig. 4). Adding HGF to the cultures resulted in a significant reduction in HNE expression ($P = .00049$). HGF showed its greatest effect at a concentration of 4 ng/mL, at which HNE production was attenuated to 59.0% of the level in the absence of HGF.

DISCUSSION

The present study has demonstrated that the application of HGF attenuates neomycin-induced hair cell death in cochlear explants. In our experimental setting, quantitative assessments of hair cell numbers following neomycin application exhibits that both IHC and OHC numbers decrease as the neomycin dose increases. In this setting we examined the effects of 20 ng/mL HGF and the results demonstrated significant effects of HGF on dose-dependent hair cell loss by neomycin, which strongly supports our hypothesis that HGF is a potent protectant for the cochlear hair cells. Also, in using a concentration of 1 mM neomycin, which provides a significant lesion to the hair cells, we showed that the optimal concentration of HGF is 20 ng/mL for hair cell protection in explant cultures.

HGF protection of neuronal cells is believed to involve various mechanisms including HGF/c-Met

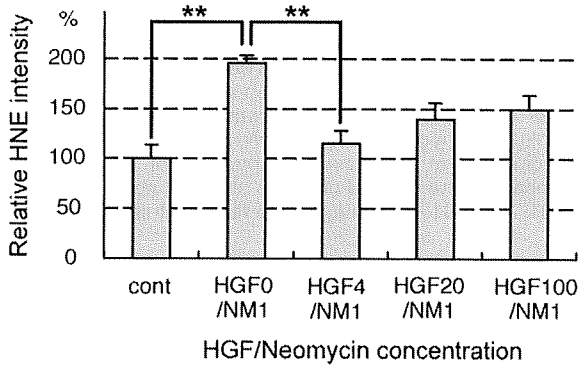

Fig. 4. Hepatocyte growth factor (HGF) treatment reduced lipid oxygenation. Lipid oxygenation was measured in cultures treated with neomycin and HGF as the intensity of immunohistochemical labeling for 4-hydroxynonenal (HNE). Relative HNE staining intensity in the organ of Corti was significantly increased in the presence of 1 mM neomycin, but significantly reduced with the addition of 4 ng/mL HGF. $^{**}P < .01$. Bars represent standard errors.

signaling.[5,6] We thus investigated involvement of HGF/c-Met signaling in hair cell protection by HGF against neomycin toxicity. Immunohistochemistry in normal P3 mouse cochleae revealed the presence of c-Met in cochlear hair cells. In addition, Western blotting demonstrated an increase of c-Met expression after neomycin-induced damage. These findings indicate involvement of paracrine coupling between HGF and c-Met in the auditory epithelium and the central nervous system.[5] Also, the increase of c-Met expression after neomycin-induced damage justifies exogenous HGF application for hair cell protection.

HGF could also protect hair cells from damage by reducing the oxidative stress generated by neomycin. Several researchers have reported that HGF exerts antioxidant activity by enhancing reactive oxygen species (ROS) scavenging and suppressing ROS production.[14,15] We have previously shown that lipid peroxidation caused by hydroxyl radicals increases in the auditory epithelium during cisplatin-induced hearing trauma.[13] In this study, our results indicated that lipid peroxidation also occurred in the neomycin-damaged cochlea, and HGF successfully attenuated HNE expression at a concentration of 4 ng/mL. We therefore consider that the antioxidant activity of HGF may in part play a role in the mechanisms by which it protects hair cells.

CONCLUSION

We have provided evidence for the direct survival-promoting effect of HGF on cochlear hair cells. Our data indicate that damaged auditory epithelia express c-Met receptors and HGF is a candidate as a delivering drug to the cochlea for treatment of sensorineural hearing loss.

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PROSTAGLANDIN E RECEPTOR SUBTYPE EP4 AGONIST PROTECTS COCHLEAE AGAINST NOISE-INDUCED TRAUMA

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Abstract—Prostaglandin E_1 is frequently used for the clinical treatment of acute sensorineural hearing loss. However, the mechanisms underlying the effects of prostaglandin E_1 on the inner ear have not yet been elucidated. The physiological effects of prostaglandin E_1 are mediated by the prostanoid receptors prostaglandin I receptor and the prostaglandin E receptor subtypes EP1, EP2, EP3, and EP4, the respective agonists for which have been purified. In the current study, we examined the efficacy of a local EP4 agonist application for the treatment of sensorineural hearing loss. We examined EP4 expression in the mouse cochlea using the reverse transcription–polymerase chain reaction and immunohistochemistry. The protective effects of local EP4 agonist treatment before or after noise exposure were tested in guinea pigs using measurements of auditory brain-stem responses and histological analysis. The results demonstrated EP4 expression in the cochlea, and showed that pre- and post-treatment with an EP4 agonist significantly attenuated threshold shifts of auditory brain stem responses, and significant attenuation in the loss of outer hair cells was found in local EP4 agonist treatment before noise exposure. These findings indicate that EP4 is involved in mechanisms for prostaglandin E_1 actions on the cochlea, and local EP4 agonist treatment could attenuate acute sensorineural hearing loss.

Key words: acoustic trauma, hair cell, hearing loss, inner ear, prostanoid.

Sensorineural hearing loss (SNHL) is one of the most frequent disabilities. Once hearing has been lost, it is rarely recovered, because the mammalian auditory system, particularly the sensory hair cells (HCs), has a limited capability for regeneration. Clinically, there are no curative therapeutic options for chronic SNHL, and the curative rate for acute SNHL is also limited. The systemic application of corticosteroids has been accepted as the primary treatment of choice for acute SNHL, although its efficacy has not been substantiated (Wei et al., 2006). In general, approximately 50% of SNHL cases show no response to the systemic application of corticosteroids (Ogawa et al., 2002). Other options for the treatment of acute SNHL have therefore been required. Prostaglandin E_1 (PGE_1) has often been used as a secondary choice treatment for acute SNHL. However, its clinical efficacy remains controversial (Ahn et al., 2005; Suzuki et al., 2008; Zhuo et al., 2008). PGE_1 is usually applied to improve local blood circulation. An experimental study using guinea pigs demonstrated an increased blood supply in the cochlea following local PGE_1 application (Tominaga et al., 2006). However, the actual mechanisms underlying the effects of PGE_1 on the inner ear have not yet been elucidated.

The physiological actions of PGE_1 are mediated by the prostanoid receptors prostaglandin I receptor (IP) and the prostaglandin E receptor subtypes EP1, EP2, EP3, and EP4 (Coleman et al., 1994; Kiriyama et al., 1997). Recently, these prostanoid receptors have been cloned, and their actions have been elucidated. IP, EP2, and EP4 are coupled to G-protein stimulation and mediate increases in cyclic AMP (cAMP) that activate protein kinase A (PKA) (Coleman et al., 1994; Narumiya et al., 1999). The EP4 receptor in particular has various physiological and pathophysiological actions, including anti-apoptotic (Kataoka et al., 2005), anti-excitotoxicity (Ahmad et al., 2005), and anti-inflammatory (Kabashima et al., 2002; Nitta et al., 2002) effects. The actions of EP4 agonists could protect auditory HCs, and might indicate therapeutic efficacy of PGE_1 for acute SNHL. We therefore investigated the potential use of an EP4 agonist in the protection of auditory HCs from noise trauma. In the current study, we demonstrated EP4 expression in the cochlea using the reverse transcription–polymerase chain reaction (RT-PCR) and immunohistochemistry. We also showed the efficacy of local EP4 agonist treatment for protecting auditory HCs against noise-induced damage, by means of auditory brain-stem response (ABR) recordings and histological analyses of cochlear specimens.

EXPERIMENTAL PROCEDURES

Experimental animals

Male C57BL/6 mice at 8 weeks of age and Hartley guinea pigs weighing 350–400 g were purchased from Japan SLC, Inc. (Hamamatsu, Japan). The Animal Research Committee of the Graduate School of Medicine, Kyoto University, Japan, approved all of the experimental protocols. Animal care was supervised by

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Abbreviations: ABR, auditory brain-stem response; bp, base pairs; cAMP, cyclic AMP; DMSO, dimethyl sulfoxide; EDTA, ethylenediamine tetra-acetic acid; EP, prostaglandin E receptor subtype; HC, hair cell; HGF, hepatocyte growth factor; IHC, inner hair cell; IP, prostaglandin I receptor; OHC, outer hair cell; PBS, phosphate-buffered saline; PFA, paraformaldehyde; PGE_1, prostaglandin E_1; PKA, protein kinase A; RT-PCR, reverse transcription–polymerase chain reaction; RWM, round window membrane; SNHL, sensorineural hearing loss; SPL, sound pressure level; VEGF, vascular endothelial growth factor.

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the Institute of Laboratory Animals of the Graduate School of Medicine, Kyoto University. All of the experimental procedures were performed in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. We intended to minimize the numbers of animals and their suffering.

EP4 mRNA expression in mouse cochleae

Under general anesthesia with midazolam (10 mg/kg; Astellas, Tokyo, Japan) and xylazine (10 mg/kg; Bayer, Tokyo, Japan), mice were sacrificed and their cochleae were immediately collected. Each mouse cochlea was homogenized, and total RNA was extracted using an RNeasy mini kit (Qiagen Ltd., Valencia, CA, USA). Complementary DNA was synthesized from DNase I-treated total RNA using the Superscript first-strand synthesis kit (Invitrogen, Carlsbad, CA, USA). Polymerase chain reaction (PCR) was performed using a GeneAmp PCR system 9700 (Perkin Elmer Applied Biosystems, Foster City, CA, USA). The primers used in the PCR for EP4 and for β-actin as an invariant control were as follows: EP4 (424 base pairs [bp]), 5′-TTCCGCTCGTGGTGCGAGTGTTC-3′ (sense) and 5′-GAGGTGGTGTCTGCTTGGGTACG-3′ (antisense); and β-actin (321 bp), 5′-ACCAACTGGGACGACATGGAGAAGATCTGG-3′ (sense) and 5′-CCGGCCAGCCAGGTCCAGACGCAGGATGGC-3′ (antisense). The PCR conditions for EP4 were as follows: denaturation at 94 °C for 48 s, annealing at 64 °C for 42 s, and extension at 72 °C for 25 s. The PCR conditions for β-actin were as follows: denaturation at 94 °C for 48 s, annealing at 62 °C for 42 s, and extension at 72 °C for 72 s. The numbers of PCR cycles were 35 and 30 for EP4 and β-actin, respectively. All reactions were confirmed to be in the logarithmic phase by monitoring the PCR products obtained at the indicated number of cycles (±2). The PCR products were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide. The RT-PCR experiments were independently repeated three times using the same total RNA material. Extracts of mouse lungs were used as positive controls, and negative controls were obtained by omitting reverse transcriptase from the reactions.

Immunohistochemistry for EP4 in the cochlea

Immunohistochemistry was performed to examine the localization of EP4 in the auditory epithelia. Cochlear specimens from the mice were fixed with 4% paraformaldehyde (PFA) in 0.01 M phosphate-buffered saline (PBS; pH 7.4) at 4 °C for 12 h. After decalcification with 0.1 M EDTA for 7 days at 4 °C, 10-μm-thick cryostat sections were prepared. Anti-EP4 receptor polyclonal antibody (dilution, 1:200; Cayman Chemical, Ann Arbor, MI, USA) was used as the primary antibody for 12 h at 4 °C, and Alexa 568–conjugated goat anti-rabbit immunoglobulin G (dilution, 1:500; Molecular Probes, Eugene, OR, USA) was used as the secondary antibody for 1 h at room temperature. After immunostaining for EP4, the nuclei were counterstained with 4,6-diamidino,2-phenylindole dihydrochloride (DAPI; 1 μg/ml in PBS; Molecular Probes). Heart specimens obtained from the mice were used as positive controls for EP4. Nonspecific labeling was tested by blocking protein–antibody complex formation using EP4 receptor blocking peptide (Cayman Chemical). The specimens were viewed with a Leica TCS-SPE confocal microscope (Leica Microsystems, Wetzlar, Germany).

Drug application and noise exposure

The EP4 agonist used was ONO-AE1-329 (Ono Pharmaceutical, Co., Ltd., Osaka, Japan). After the ABR measurements, the otic bulla of the left temporal bone of each guinea pig was exposed using a retroauricular approach under general anesthesia with midazolam (10 mg/kg i.m.) and xylazine (10 mg/kg i.m.). A small hole was made in the left bulla to expose the round window niche. A gelatin sponge in dry conditions was cut into pieces 1.5–2 mm^3 in size under microscopy. A piece of gelatin that had been immersed in the EP4 agonist, which had been dissolved in dimethyl sulfoxide (DMSO) and diluted with physiological saline to give a final concentration of 1 mg/ml containing 1% DMSO, was then placed on the round window membrane (RWM) of the animals in the EP4 agonist group (n=8). For the animals in the control group, a piece of gelatin that had been immersed in physiological saline containing 1% DMSO was used (n=9). The animals were exposed to one octave band noise centered on 4 kHz at a sound pressure level (SPL) of 120 dB for 5 h in a ventilated sound-exposure chamber immediately after drug application under general anesthesia with midazolam and xylazine. Each animal was immobilized, and a speaker was centered over the animal's head at a distance of 15 cm. The sound chamber was fitted with speakers driven by a noise generator and a power amplifier. Using a 1/2-inch condenser microphone (Sony, Tokyo, Japan) and fast Fourier transform analyzer (Sony), sound levels were monitored and calibrated at multiple locations within the sound chamber to ensure uniformity of the stimulus. The stimulus intensity varied by a maximum of 3 dB SPL across measured sites within the exposure chamber. We also examined post-treatment effects. The animals were locally administered an EP4 agonist (n=5) or physiological saline containing 1% DMSO (n=5) 30 min after noise exposure. ABR thresholds following local application of saline containing 1% DMSO were measured in normal guinea pigs (n=4) as a control experiments.

ABR measurements

The ABR thresholds were measured at frequencies of 4, 8, and 16 kHz before noise exposure, and on days 3, 7, 14 and 21 after drug application. The animals were anesthetized with midazolam and xylazine, and kept warm with a heating pad. The generation of acoustic stimuli and the subsequent recording of evoked potentials were performed using a Powerlab/4sp (ADInstruments, Colorad Springs, CO, USA). The acoustic stimuli, which consisted of tone-burst stimuli (0.1 ms cos2 rise/fall and 1-ms plateau), were delivered monaurally through a speaker (ES1spc; Bioresearch Center, Nagoya, Japan) connected to a funnel fitted into the external auditory meatus. To record bioelectrical potentials, subdermal stainless-steel needle electrodes were inserted at the vertex (ground), ventrolateral to the measured ear (active), and contralateral to the measured ear (reference). The stimuli were calibrated against a 3-inch free-field microphone (ACO-7016; ACO Pacific, Belmont, CA, USA) connected to an oscilloscope (DS-8812 DS-538; Iwatsu Electric, Tokyo, Japan) or a sound level meter (LA-5111; Ono Sokki, Yokohama, Japan). The thresholds were determined from a set of responses at varying intensities with 5-dB SPL intervals, and the electrical signals were averaged over 1024 repetitions. The thresholds at each frequency were verified at least twice.

Histological analyses

On day 21 after drug application, the temporal bones were collected and immersed in 4% PFA in 0.01 M PBS at 4 °C for 12 h. After decalcification with 0.1 M EDTA for 14 days at 4 °C, the cochleae were subjected to histological analysis as whole mounts. Three regions of the cochlear sensory epithelia, at a distance of 30%–50% (corresponding to 1–3 kHz regions, second turn), 50%–70% (corresponding to 3–8 kHz regions, mid-basal portion), and 70%–90% (corresponding to 8–30 kHz regions, basal portion) from the apex (Viberg and Canlon, 2004), were used for quantitative assessments of HC loss. Cochlear specimens were permeabilized in 0.2% Triton X in PBS for 30 min at room temperature. Immunohistochemistry for myosin VIIa and F-actin labeling by phalloidin were used to label the inner hair cells (IHCs) and the outer hair cells (OHCs). Anti-myosin VIIa rabbit polyclonal antibody (dilution, 1:500; Proteus BioSciences, Ramona, CA, USA)

was used as the primary antibody for 12 h at 4 °C, and Alexa 568–conjugated goat anti-rabbit immunoglobulin G (dilution, 1:500) was used as the secondary antibody for 1 h at room temperature. After immunostaining for myosin VIIa, the specimens were stained with fluorescein–phalloidin (1:400; Molecular Probes) for 15 min at room temperature. Specimens were viewed under confocal microscopy (TCS SP2; Leica Microsystems, Wetzlar, Germany). Nonspecific labeling was tested by omitting the primary antibody from the staining procedures. The numbers of IHCs and OHCs in 0.2-mm-long regions of each region of the cochleae were counted by the three authors (R.H., T.N., and T.S.). The average of these values was used for each animal. The samples obtained from eight animals pre-treated with an EP4 agonist, nine animals pre-treated with saline containing DMSO, five animals post-treated with an EP4 agonist and five animals post-treated with saline containing DMSO were provided for statistical analyses.

Statistical analysis

The overall effects of EP4 agonist application on ABR threshold shifts were examined using two-way factorial analysis of variance. When the interactions were significant, multiple comparisons using the Fisher protected least significant difference test were performed for pairwise comparisons. Differences in the numbers of IHCs and OHCs in each region between the EP4 agonist-treated cochleae and the control cochleae were examined using the Student's *t*-test. *P*-values <0.05 were considered statistically significant. Values are expressed as the mean and the standard error of the mean (SEM).

RESULTS

EP4 expression in cochleae

RT-PCR analysis of the EP4 mRNA levels was performed to investigate EP4 expression. Amplification of β-actin, yielding a 321-bp amplicon, was used as an internal control (Fig. 1). Negative control reactions that lacked reverse transcriptase failed to yield amplicons of either β-actin or EP4 (lane 3 in Fig. 1). We used the mouse lung as a positive control for EP4, which exhibited EP4 mRNA expression (lane 1 in Fig. 1). The mouse cochleae also demonstrated EP4 mRNA expression (lane 2 in Fig. 1). Immunostaining revealed that EP4 expression occurred in the stria vascularis, spiral ligament, spiral ganglion neurons, supporting cells, and HCs (Fig. 2A–C), while negative controls using a specific blocking peptide showed no immunoreactivity (Fig. 2D–F). These findings confirmed that EP4 expression was present in the cochlear cells, including the HCs.

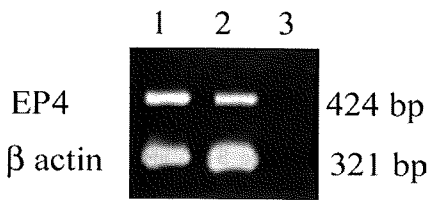

Fig. 1. RT-PCR for EP4 and β-actin expression in the inner ear. EP4 (424 bp band) was detected in mouse lung (lane 1) and cochleae (lane 2) specimens. β-Actin (321 bp band), as an invariant control, was detected in both types of specimen. No product was detected in specimens in the absence of the RT reaction (lane 3).

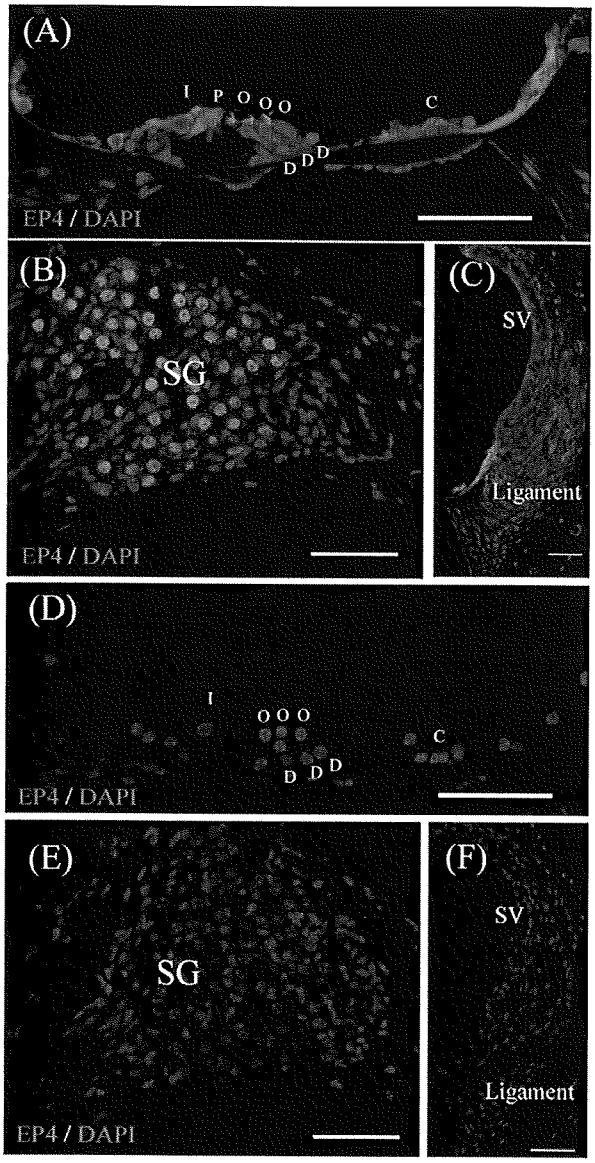

Fig. 2. Immunoreactivity for EP4 and counterstained nuclei. (A–C) EP4 expression was detected in the stria vascularis (SV), spiral ganglion cells (SG), sensory epithelium (I, IHC; O, OHC; D, Deiter's; C, Claudius' cell; P, pillar cell). In negative controls that were stained after blocking with specific peptide (D–F), no immunoreactivity for EP4 is found in the cochlea. Scale bar=200 μm.

ABR threshold shifts

Local application of saline containing 1% DMSO in normal guinea pigs showed no significant elevation of ABR thresholds following drug application (data not shown). The time courses of alterations in the ABR threshold shifts in noise-exposed animals at 4, 8, and 16 kHz are shown in Fig. 3. For pre-treatment experiments, the data from eight animals treated with the EP4 agonist and that from nine animals treated with physiological saline containing DMSO were provided for statistical analyses. Local EP4 agonist treatment had significant effects on the ABR threshold shifts at each frequency. The overall effect of EP4 agonist application at 4, 8, and 16 kHz was significant ($P<0.0001$).

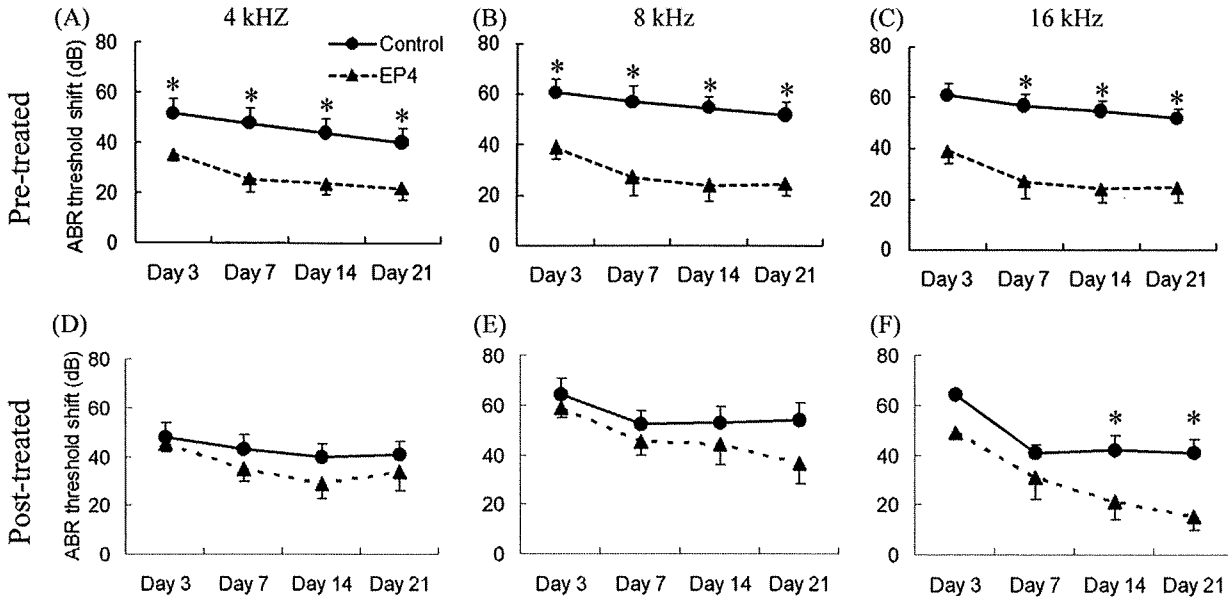

Fig. 3. Time courses of alterations in threshold shifts of ABRs in EP4 agonist-treated cochleae and control cochleae at frequencies of 4, 8, and 16 kHz in pre-treated animals (A–C) or in post-treated animals (D–F). In pre-treated animals, the overall effects at 4, 8, and 16 kHz of EP4 agonist application were significant ($P<0.0001$). The differences in ABR threshold shifts at 4 and 8 kHz between EP4 agonist-treated cochleae and control cochleae on days 3, 7, 14 and 21 were significant. The differences in ABR threshold shifts at 16 kHz on days 7, 14 and 21 were significant (* $P<0.05$). In post-treated animals, the overall effects at 8 and 16 kHz of EP4 agonist application were significant ($P=0.042$ for 8 kHz and $P<0.0001$ for 16 kHz). The differences in ABR threshold shifts at 16 kHz on days 14 and 21 were significant (* $P<0.05$). Bars represent the SEM.

The differences in the threshold shifts at 4 kHz between the EP4 agonist-treated cochleae and the control cochleae on days 3, 7, 14 and 21 were shown to be significant in multiple comparisons using the Fisher protected least significant difference test ($P=0.039$ for day 3, $P=0.005$ for day 7, $P=0.010$ for day 14, and $P=0.020$ for day 21). The differences in the threshold shifts at 8 kHz between the EP4 agonist-treated cochleae and the control cochleae were significant on days 3, 7, 14 and 21 ($P=0.008$ for day 3, $P<0.001$ for day 7, $P=0.010$ for day 14, and $P=0.001$ for day 21). The differences in the threshold shifts at 16 kHz between the EP4 agonist-treated cochleae and the control cochleae were significant on days 7, 14 and 21 ($P=0.017$ for day 7, $P=0.012$ for day 14, and $P=0.011$ for day 21), but not on day 3 ($P=0.117$). For post-treatment experiments, the data from five animals treated with the EP4 agonist and that from five animals treated with physiological saline containing DMSO were provided for statistical analyses. The overall effects of EP4 agonist application at 8 and 16 kHz were significant ($P=0.042$ for 8 kHz and $P<0.0001$ for 16 kHz), but not at 4 kHz. The differences in the threshold shifts at 16 kHz between the EP4 agonist-treated cochleae and the control cochleae on days 14 and 21 were shown to be significant in multiple comparisons ($P=0.008$ for day 14 and $P=0.003$ for day 21). No significant difference in ABR threshold shifts at 8 kHz was found in multiple comparisons. These findings indicate that local EP4 agonist treatment offered functional protection of the auditory system against noise-induced damage.

HC protection

Immunostaining for myosin VIIa and phalloidin staining demonstrated severe degeneration of the OHCs in the second turn, mid-basal, and basal portions of the control cochleae (Fig. 4A, D, G); by contrast, the OHC degener-

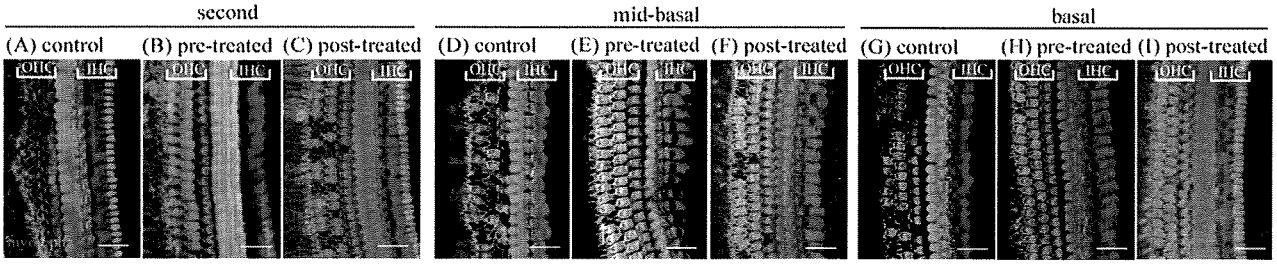

Fig. 4. Immunostaining for myosin VIIa (myo; red) and F-actin labeling with phalloidin (pha; green) of cochlear sensory epithelia in the second turn, mid-basal and basal portions. Severe loss of OHCs was observed in the control cochlea (A, D, G). Degeneration of OHCs was limited in the specimen pre-treated with an EP4 agonist (B, E, H). Moderate degeneration of OHCs was observed in the second turn and the mid-basal portion of the cochlea post-treated with an EP4 agonist (C, F, I). Scale bar=25 μm. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

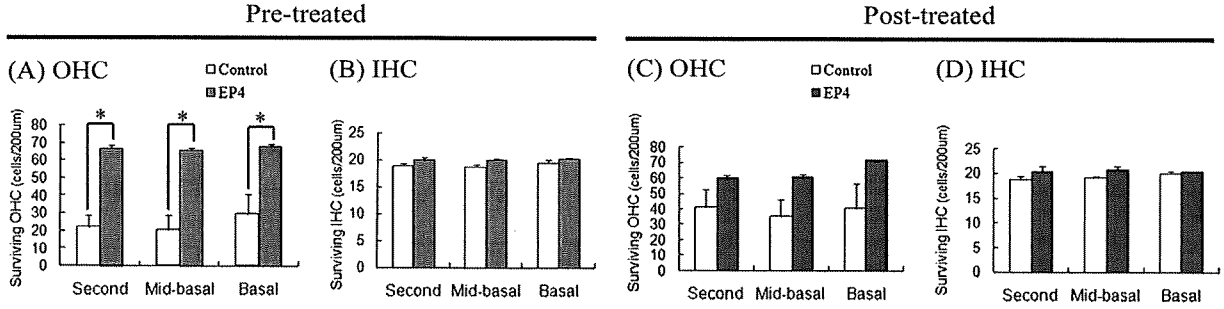

Fig. 5. Numbers of surviving OHCs and IHCs in the second turn, mid-basal and basal portions apical, middle, and basal portions of cochleae. Significant differences in the numbers of surviving OHCs were found between the EP4 agonist-treated cochleae and the control cochleae in all the portions in a pre-treated condition (A; * $P<0.01$). However, in a post-treated condition, significant differences in the numbers of OHCs were not found between the two groups (C). No significant differences in the numbers of IHCs were found between the two groups in either condition (B, D). Bars represent the SEM.

ation was limited in the cochleae pre-treated with an EP4 agonist (Fig. 4B, E, H). The IHCs were preserved in both experimental groups (Fig. 4A, B). Quantitative assessments revealed significant differences in the numbers of surviving OHCs in all cochlear portions between the cochleae pre-treated with an EP4 agonist and those pre-treated with saline containing DMSO (Fig. 5A). The differences in the numbers of surviving OHCs between the EP4 agonist-treated cochleae and the control cochleae were significant in the second turn ($P<0.001$), mid-basal ($P<0.001$), and basal portions ($P=0.006$). No significant differences were observed in the numbers of surviving IHCs between the EP4 agonist-treated cochleae and the control cochleae (Fig. 5B). In post-treatment experiments, the OHC degeneration in the cochleae post-treated with an EP4 agonist appeared to be moderate (Fig. 4C, F, I), however, quantitative assessments revealed no significant differences between the EP4 agonist-treated cochleae and the control cochleae (Fig. 5C). No significant differences in the numbers of surviving IHCs were also observed between the two groups in post-treatment experiments (Fig. 5D).

DISCUSSION

PGE_1 has been applied to patients with idiopathic sudden SNHL; however, its clinical efficacy and its mechanism of action for this disease have not been determined. PGE_1 directly stimulates several signaling pathways via the PGI receptor (IP) and four subtypes of the PGE receptors (EP1-4), resulting in changes cAMP and phosphoinositide cellular levels (Kiriyama et al., 1997; Narumiya et al., 1999). The present results show the expression of EP4 in the cochlea, and the attenuation of noise-induced damage by local EP4 agonist administration, suggesting an involvement of EP4 in mechanisms for therapeutic effects of PGE_1 for SNHL. On the other hand, IP, EP2 and EP4 mediate an increase of intracellular cAMP levels, while the activation of the EP3 leads to a decrease of cAMP levels. Therefore, the stimulation of EP3 by PGE_1 may contradict effects of IP, EP2 or EP4 stimulation by PGE_1, indicating that selective antagonists for IP, EP2 or EP4 might be more effective for treatment of SNHL than PGE_1.

Recent studies have indicated the potential of EP4 agonists for neuro-protection. An *in vitro* study using primary culture of cortical neurons has revealed that EP4 agonists protect neurons against amyloid β-peptide toxicity via the cAMP–PKA pathway (Echeverria et al., 2005), which is also involved in mechanisms for promotion of the auditory neuron survival (Bok et al., 2003; Alam et al., 2007). An EP4 agonist, ONO-AE1-329, which was used in the present study, has been reported to attenuate brain injury due to glutamate excitotoxicity *in vivo* (Ahmad et al., 2005). Excitotoxicity mediated by glutamate receptors also plays an important role in mechanisms of cochlear damage induced by noise trauma and ischemic injury (Pujol et al., 1999; Ruel et al., 2007). These previous findings encouraged us to investigate protective effects of ONO-AE1-329 against noise trauma. As we expected, present findings demonstrate that local EP4 agonist treatment applied to the cochlea significantly reduced the elevation of the ABR thresholds and OHC loss, indicating the potential of an EP4 agonist, ONO-AE1-329, for cochlea protection similarly to the CNS.

There are several potential explanations for the mechanisms underlying otoprotection by EP4 agonists. One possible mechanism is a direct action of EP4 agonists on cochlear HCs. The stimulation of EP4 in HCs may induce an increase of intracellular cAMP levels, which activates the PKA pathway resulting in promotion of the HC survival. The regulation of growth factors such as vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) in the cochlea by EP4 agonists is also included in possible mechanisms for HC protection. Numerous previous reports have demonstrated that PGE increases VEGF expression (Bamba et al., 2000; Pai et al., 2001; Weiss et al., 2004) and HGF production (Makino et al., 2004; Zhang et al., 2000). Hatazawa (2007) demonstrated that PGE_2 stimulates VEGF expression through EP4 receptors. The upregulation of VEGF in the inner ear is involved in the recovery of auditory function after acoustic trauma, with potentially important clinical and therapeutic implications (Picciotti et al., 2006). HGF gene transfer to the subarachnoid space using virus vectors can prevent or ameliorate hearing impairment (Oshima et al., 2004). In addition, local

HGF protein application to the cochlea attenuates noise-induced damage on the cochlea (Inaoka et al., 2009). Such mechanisms might be involved in the EP4 agonist-induced protection of auditory HCs against injury caused by exposure to noise. The recovery of the circulation in the cochlea is also included in potential explanations for otoprotection by EP4 agonists. Local application of PGE_2 onto the RWM induces an increase of blood flow in the cochlea (Rhee et al., 1999). Exposure to intense noise causes reduction of the cochlear blood flow (Nakashima et al., 2003). Therefore, local EP4 treatment might improve a decrease of the cochlear blood flow due to noise exposure. Further studies are required to elucidate the detailed mechanisms underlying the EP4 agonist-induced protection of auditory HCs.

CONCLUSION

The present findings demonstrate the expression of EP4 in the cochlea, and cochlear protection against noise trauma by local EP4 agonist application to the RWM. The elevation of the ABR threshold was significantly reduced, and histological analysis revealed that pre-treatment with an EP4 agonist significantly reduced the loss of OHCs. EP4 agonists might therefore have potential applications in clinical treatments against noise-induced hearing loss. However, the exact mechanisms by which EP4 agonists act in the cochlea are presently unclear and require further research.

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Hydrogen protects auditory hair cells from free radicals

Yayoi S. Kikkawa, Takayuki Nakagawa, Rie T. Horie and Juichi Ito

Reactive oxygen species (ROS) play a role in the degeneration of auditory hair cells because of aging, noise trauma, or ototoxic drugs. Hydrogenation is a fundamental reduction/deoxidation reaction in living organisms. This study thus examined the potential of hydrogen to protect auditory hair cells from ROS-induced damage. To generate ROS, we applied antimycin A to explant cultures of auditory epithelia, and examined the effect of hydrogen on the protection of hair cells against ROS. Incubation with a hydrogen-saturated medium significantly reduced ROS generation and subsequent lipid peroxidation in the auditory epithelia, leading to increased survival of the hair cells. These findings show the potential of hydrogen to protect auditory hair cells from ROS-induced damage. *NeuroReport* **20:689–694 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Keywords: antioxidant, cochlea, hearing loss, hydroxyl radical, molecular hydrogen, reactive oxygen species

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Introduction

Hearing disorders affect nearly 10% of the world population. The common causes of sensorineural hearing loss because of cochlear injury are aging, hereditary disorders, noise trauma, and ototoxic drugs. The mechanisms underlying cochlear injury are still not completely known. However, numerous studies have suggested that they involve the production of reactive oxygen species (ROS), which cause cellular injury in the cochlea resulting in sensorineural hearing loss [1–4]. Although they are probably intended to fight against invasive pathogens, ROS seem to produce substantial collateral damage through DNA strand breaks, lipid and protein oxidation [5–7].

Hydrogenation is a fundamental reduction/deoxidation reaction in living organisms. Many reduction processes in the body involve electron transfer from molecular hydrogen. This molecule was recently established as an antioxidant that selectively reduces the hydroxyl radical, and was shown to decrease the cerebral infarction volume after ischemia in rats [8]. Subsequently, protective effects of hydrogen gas have been demonstrated in a mouse model for hepatic injury [9] and in a rat model for myocardial infarction [10]. In the nervous system, hydrogen-rich water was shown to prevent superoxide formation in mouse brain slices [11], and to prevent stress-induced impairments in learning tasks during chronic physical restraint in mice [12]. Moreover, a clinical study showed that consuming hydrogen-rich pure water improves lipid and glucose metabolism in type 2 diabetes patients [13].

The ex-vivo study reported here tested the hypothesis that molecular hydrogen, hydrogen gas, protects against cochlear impairment by scavenging free radicals. We initially generated in situ ROS in the cochlea using an inhibitor of mitochondrial respiratory chain complex III, antimycin A, and showed that they caused direct damage to the hair cells. Then, using a hydrogen (hydrogen gas)-saturated culture media, we demonstrated that hydrogen gas alleviated ROS-induced ototoxicity, suggesting that hydrogen gas has the potential to act as an antioxidant for the treatment of cochlear damage. We also evaluated the generated hydroxyl radicals by fluorescence emission of 2-[6-(4′-hydroxy)phenoxy-3H-xanthen-3-on-9-yl] benzoate (HPF) and lipid peroxidation by immunohistochemistry for 4-hydroxynonenal (HNE).

Materials and methods

Animals

The ICR mice (Japan SLC, Hamamatsu, Japan) used in this study were cared for in the Institute of Laboratory Animals of the Kyoto University Graduate School of Medicine. The Animal Research Committee of the Kyoto University Graduate School of Medicine approved all experimental protocols, which were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Cochlear explant culture

Postnatal day 2 (P2) ICR mice were deeply anesthetized with diethyl ether and decapitated. The temporal bones were dissected, and the cochleae were freed from the surrounding tissue and placed in 0.01 M phosphate-buffered saline (PBS; pH 7.4). After removing the cochlear lateral wall, the auditory epithelia were dissected from the cochlear modiolus. The tissue samples were then placed on glass-mesh inserts (Falcon, Billerica, Massachusetts, USA) and cultured initially in serum-free

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modified Eagle's medium (MEM; Invitrogen, Eugene, Oregon, USA), supplemented with 3 g/l glucose (Wako Pure Chemicals, Osaka, Japan) and 0.3 g/l penicillin G (Wako), for 24 h at 37°C in a humidified (95%) air: 5% atmospheric CO_2. In total, 20 cochlear explants were used in a single culture, and at least three independent cultures were performed for each condition. As the hair cells in the apex are resistant to free radicals [14], the basal turns of the cochlea were used in this study.

Antimycin A application

The explants were transferred to medium containing antimycin A (Sigma–Aldrich, St Louis, Missouri, USA) at concentrations of 0.1, 1, or 10 μg/ml, with six to nine cochleae incubated at each concentration. The cultures were maintained for 24 h. At the end of the culture period, the samples were fixed for 15 min in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), and then provided for immunostaining for myosin VIIa to evaluate the number of surviving hair cells. The specimens were incubated with primary rabbit polyclonal antibodies against myosin VIIa (1 : 500; Proteus Bioscience Inc., Ramona, California, USA). Alexa-Fluor 568 goat anti-rabbit IgG (1 : 200; Invitrogen) was used as the secondary antibody. Specimens were then incubated in Alexa-Fluor 488-conjugated phalloidin (1 : 250; Invitrogen) to label F-actin. The specimens were examined with a Leica TCS-SP2 laser-scanning confocal microscope (Leica Microsystems Inc., Wetzlar, Germany). To quantify the hair-cell loss in the cochlea after the different treatments, inner hair cells (IHCs) and outer hair cells (OHCs) were counted over a 100-μm-long stretch of the auditory epithelia, in two separate regions of the basal turn in each culture (totaling 200 μm). For each treatment, six to nine cultures were evaluated.

Hydrogen treatment

To assess the efficacy of hydrogen gas for cochlear protection, explants were cultured initially in an airtight box (Chopla Industries, Inazawa, Japan) with reduced-CO_2-dependence media, MEM and Leivovitz's L-15 media (Invitrogen) mixed in a 1 : 1 ratio [15], supplemented with 3 g/l glucose and 0.3 g/l penicillin G, at 37°C in humidified (100%) atmospheric air. After 24 h, the medium was changed to one containing antimycin A at a concentration of 0.1, 1 or 10 μg/ml, with or without hydrogen gas for another 24 h. Hydrogen gas was dissolved directly into the media, and a high content of dissolved hydrogen (1.3 ± 0.1 mg/l) was confirmed using a hydrogen electrode (Model M-10B2; Able Corporation, Tokyo, Japan). The pH of the culture media without hydrogen gas was 7.18 ± 0.02, and that of the culture media with hydrogen gas was 7.52 ± 0.02. The prepared media were used for culture within 30 min. At the end of the experiments, the explants were fixed and provided for histological analysis to evaluate hair-cell survival. Between six and 12 cochleae were used for each condition.

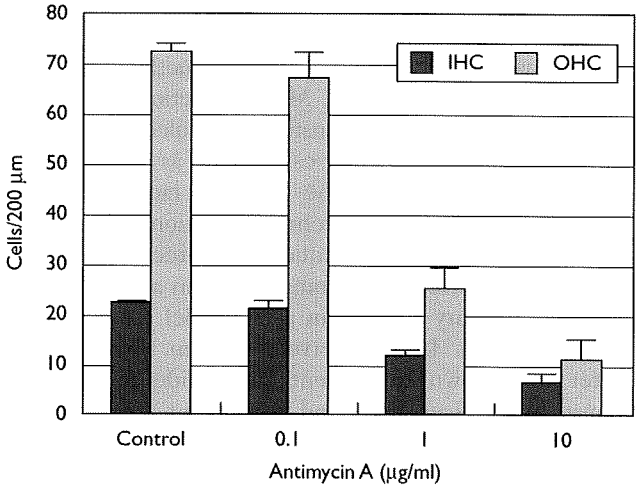
Detection of reactive oxygen species by fluorescent indicators

The cochlear explants were treated with 30 μM HPF (Daiichi Pure Chemicals Co., Tokyo, Japan) for 20 min to detect cellular hydroxyl radicals. Fluorescent images were captured with a Leica TCS-SP2 confocal microscope using excitation and emission filters of 488 and 510 nm, respectively. All images were taken with the same laser intensity, detector gain, and offset values. Fluorescent signals were quantified from two separate OHC regions of the basal turns, each of which was 1250 μm^2 (50 × 25 μm), using ImageJ software (*http://rsb.info.nih.gov/ij/*; NIH, Bethesda, MD). Intensity measurements were expressed relative to the levels in the control samples.

Lipid-peroxidation assay

Lipid peroxidation was assessed in cultures treated with antimycin A, in the presence or absence of hydrogen gas, by measuring the expression of HNE immunohistochemically. Explants were labeled with mouse anti-HNE monoclonal antibody (1 : 8; Oxis Research, Portland, Oregon, USA) and fluorescein horse anti-mouse immunoglobulin G (1 : 250; Vector Laboratories, Burlingame, California, USA) as the primary and secondary antibodies, respectively. Specimens were then counterstained with Alexa 568 phalloidin (1 : 250; Invitrogen). All images were taken with the same exposure and shutter speed. The green fluorescence intensity was measured in the same area using ImageJ software. Intensity measurements were expressed relative to the levels in the control samples.

Fig. 1



Antimycin A induced dose-dependent auditory hair-cell loss. The graph shows the relationship between the antimycin A concentration and the hair-cell count following 24-h culture. The inner hair cells (IHCs) and outer hair cells (OHCs) were counted in 200-μm-length regions from each cochlea. The hair-cell densities decreased systematically as the antimycin A concentration increased. Bars represent standard errors.

Statistical analysis

The overall effects on the hair-cell number, and the HPF and HNE staining intensities, were analyzed by two-way factorial analysis of variance (ANOVA) using the Statcel2 application (OMS Publishing, Saitama, Japan). *P* values less than 0.05 were considered to be statistically significant. For interactions that were found to be significant, multiple paired comparisons were analyzed using the Tukey–Kramer test.

Results

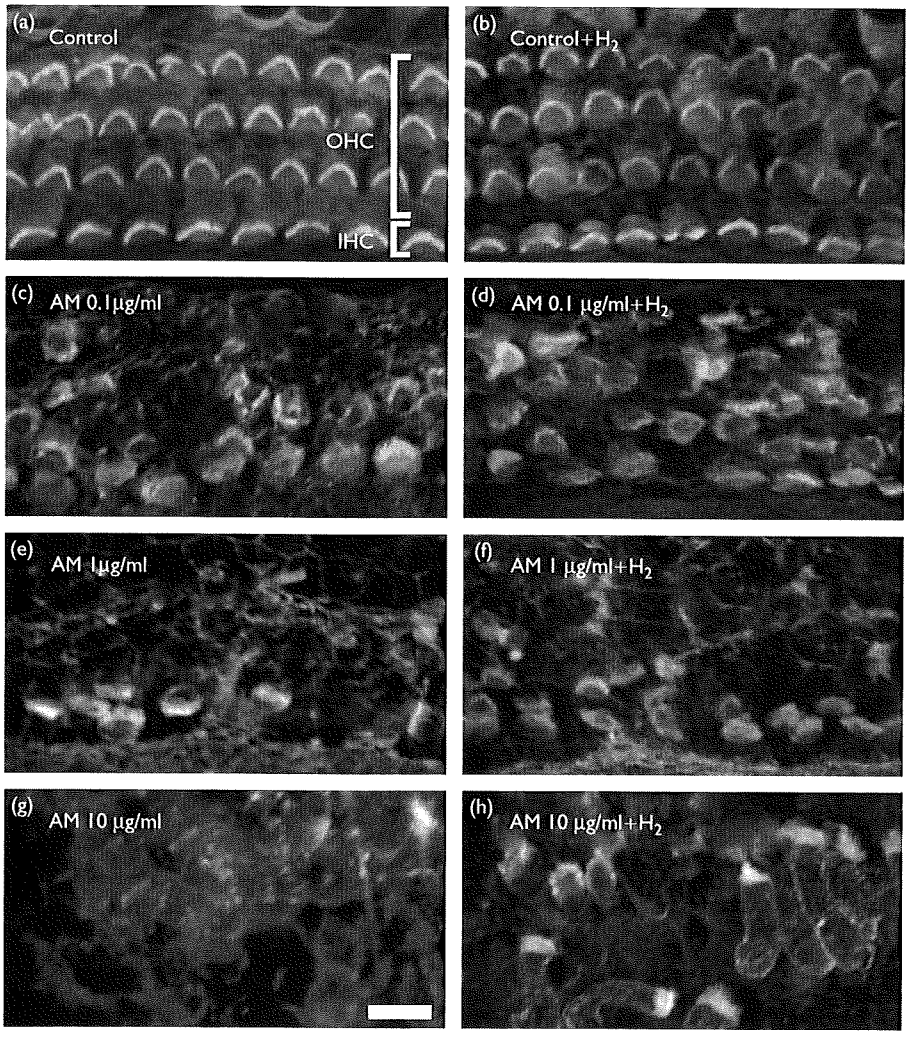
Antimycin A induced dose-dependent hair-cell loss

Initially, we established the dose–response relationship between the antimycin A concentration and its toxic effect on hair cells. The addition of 1 μg/ml antimycin A to cultures for 24 h significantly reduced the hair-cell numbers in both the IHC and OHC regions, with the effect being more severe in the latter (Fig. 1). The addition of 1 μg/ml antimycin A destroyed 46.2 ± 4.6% of the IHCs and 65.6 ± 5.8% of the OHCs. The hair-cell density decreased depending on the concentrations of antimycin A and few could be detected in the auditory epithelia cultured in 10 μg/ml antimycin A (Fig. 2g).

Protective effect of hydrogen supplementation

Next, we assessed the potential of hydrogen to protect against antimycin-induced ototoxicity by administering 0, 0.1, 1, or 10 μg/ml antimycin to samples cultured in hydrogen-saturated media in an airtight environment. The addition of hydrogen markedly increased both IHC and OHC survival, with a substantial number of hair cells surviving even at the highest antimycin A dose (Figs 2 and 3). Two-way ANOVA showed that hydrogen gas had a significant effect on the numbers of surviving IHCs and OHCs ($P = 0.00305$ and $P = 0.00016$, respectively). Tukey–Kramer tests for multiple paired comparisons

Fig. 2



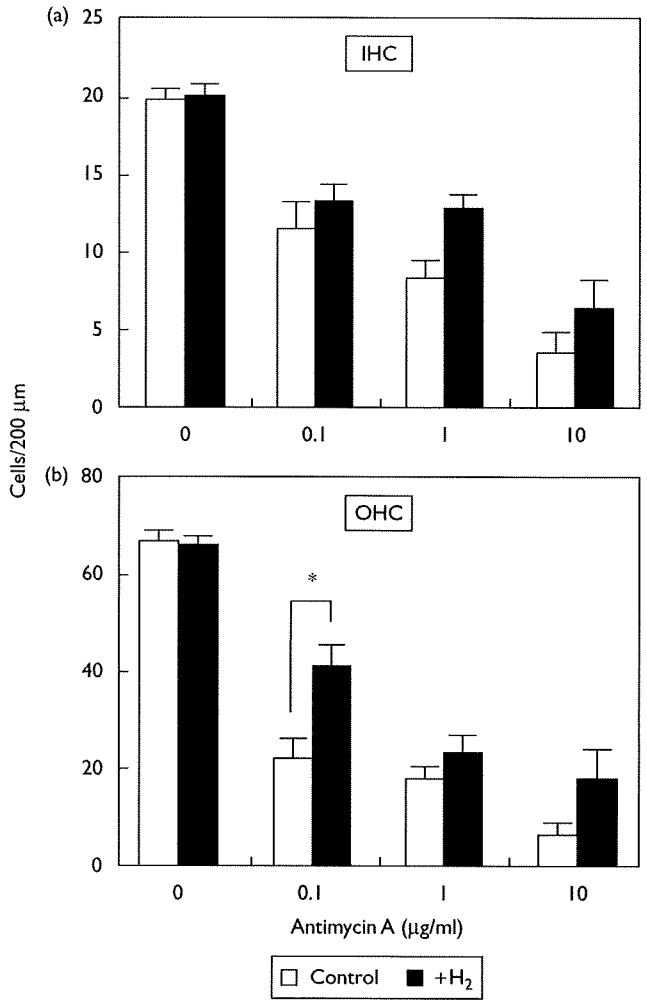
Effect of hydrogen on the survival of cochlear hair cells. (a–h) Photomicrographs of phalloidin (green) and myosin VIIa (red)-labeled cochlear cultures, treated with 0 (a and b), 0.1 (c and d), 1 (e and f), and 10 μg/ml (g and h) antimycin A (AM), with (b, d, f and h) or without (a, c, e and g) hydrogen gas (H_2). Bar, 5 μm. IHCs, inner hair cells; OHCs, outer hair cells.

showed that the loss of OHCs was significantly lower ($P < 0.01$) in the groups treated with 0.1 μg/ml antimycin A and hydrogen than in the groups treated with antimycin A alone (Fig. 3). These data showed that hydrogen protected hair cells against antimycin A-induced toxicity in this model of cochlear damage.

Reactive oxygen species reduction by molecular hydrogen

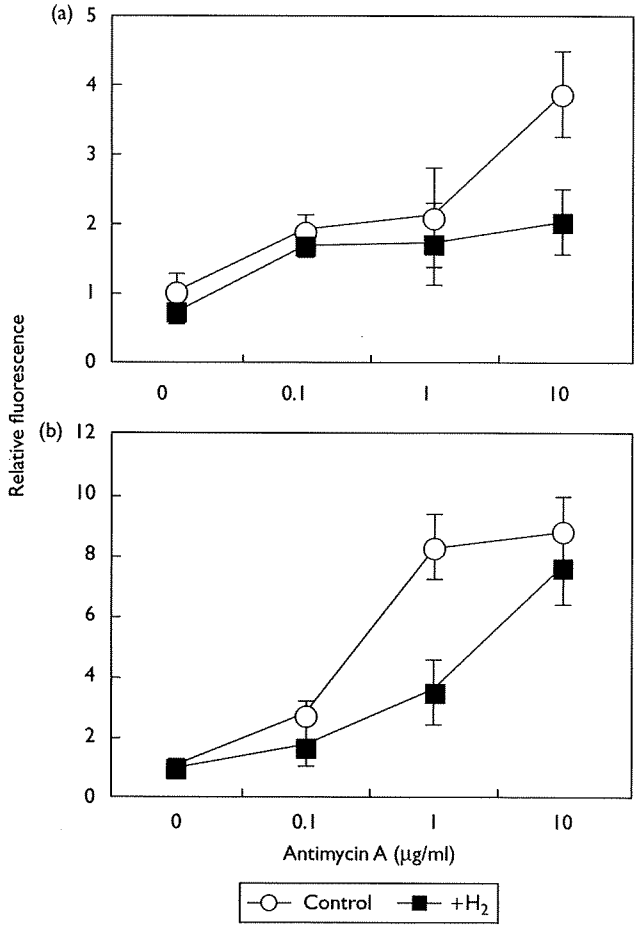
To investigate the mechanism by which hydrogen alleviated hair-cell damage, we measured the ROS production in the cochlear cultures (Fig. 3). HPF is a reagent that was developed to detect certain highly ROS directly [16]. In cochlear cultures treated with 0–10 μg/ml antimycin A for 45 min, in the absence of hydrogen gas, the HPF signals increased (Fig. 4a), indicating that antimycin A induced the production of hydroxyl radicals. The intensity of the HPF fluorescence after the treatment with 10 μg/ml antimycin A was 3.61 times greater than that in the absence of antimycin A (Fig. 4a). By contrast, adding hydrogen gas to the cultures resulted in a reduction of the HPF signal intensity.

Fig. 3



Hair-cell counts in molecular hydrogen-treated cultures. After 24-h culture with antimycin A, inner hair cells (IHCs) (a) and outer hair cells (OHCs) (b) were counted. White box symbols represent the counts from control cultures without molecular hydrogen, and black symbols represent those from cultures with molecular hydrogen. Molecular hydrogen significantly attenuated the loss of IHCs ($P=0.0031$) and OHCs ($P=0.0016$) in antimycin A-damaged cochleae according to a two-way analysis of variance. Post hoc analyses with Tukey–Kramer tests for multiple paired comparisons showed that the OHC loss was significantly lower in cultures treated with antimycin A plus hydrogen gas than in those treated with 0.1 μg/ml antimycin A alone (*$P<0.01$). Bars represent standard errors.

Fig. 4



Molecular hydrogen reduced reactive oxygen species (ROS) production and lipid oxygenation. White circle symbols represent the counts from control cultures without molecular hydrogen, and black box symbols represent those from cultures with molecular hydrogen. (a) ROS production was measured in cultures treated with antimycin A with or without hydrogen gas for 40 min, according to the intensity of chemifluorescence of 2-[6-(4′-hydroxy)phenoxy-3H-xanthen-3-on-9-yl] benzoate (HPF). The relative HPF fluorescence intensity in the organ of Corti increased dose dependently in the presence of antimycin A, but was significantly reduced by the addition of hydrogen gas ($P=0.0439$). (b) Lipid oxygenation was measured in cultures treated with antimycin A with or without hydrogen gas for 24 h, according to the intensity of immunohistochemical labeling for 4-hydroxynonenal (HNE). The relative HNE-staining intensity in the organ of Corti increased dose dependently in the presence of antimycin A, but was significantly reduced by the addition of hydrogen gas ($P=0.0447$). Bars represent standard errors.

In the presence of hydrogen gas, the intensity of the HPF fluorescence after the treatment with 10 μg/ml antimycin A was 2.34 times greater than that in the absence of antimycin A (i.e. 52.1% of that without hydrogen). Two-way ANOVA showed that hydrogen had a significant effect on the production of hydroxyl radicals ($P = 0.0439$). No significant differences were identified in multiple paired comparisons with Tukey–Kramer tests.

We also investigated the expression of HNE, which is a lipid-peroxidation marker, in the explant cultures. HNE production increased dose dependently in the presence of antimycin A. The intensity of the HNE fluorescence after the treatment with 10 μg/ml antimycin was 8.68 times greater than that in the absence of antimycin A (Fig. 4b). Adding molecular hydrogen to the cultures resulted in a significant reduction of HNE expression. Two-way ANOVA showed that hydrogen had a significant effect on the production of HNE ($P = 0.0446$), but, no significant differences were identified in multiple paired comparisons with Tukey–Kramer tests. Molecular hydrogen had its greatest effect at an antimycin concentration of 1 μg/ml, when the HNE production was attenuated to 42.4% of the level seen in the absence of molecular hydrogen.

Discussion

To our knowledge, this study was the first to evaluate the therapeutic potential of molecular hydrogen for the auditory system. A quantitative assessment of the hair-cell loss caused by antimycin A showed a dose-dependent effect, indicating that the toxic effects in this explant culture system represented a good model for the cochlea *in vivo*. Treating the cultures with hydrogen gas significantly influenced the dose response for hair-cell loss because of antimycin A, indicating that hydrogen gas has a protective effect on hair cells against ROS toxicity.

We also investigated the mechanisms by which hydrogen gas protected hair cells from damage in the cochlea. Our results showed that the hydrogen gas in fact reduced the production of cellular ROS and subsequent lipid oxygenation. Our antimycin A cochlea culture system, along with chemiluminescence detection, was shown to be useful in screening for antioxidant drugs, because antimycin A directly produces ROS in the cochlea and the direct measurement of ROS was possible when HPF was used.

Hydrogen is one of the most abundant and well-known molecules. Inhalation of hydrogen gas has been used in the prevention of decompression sickness in divers and has shown a good safety profile [8]. Hydrogen has been approved by the US Food and Drug Administration for the treatment of several different diseases. Ohsawa *et al.* [8] demonstrated that hydrogen gas is a potent antioxidant with certain unique properties. First, hydrogen gas is permeable to cell membranes and can target organelles, including mitochondria and nuclei. Second, hydrogen gas specifically quenches detrimental ROS, such as the hydroxyl radical and peroxynitrite, while maintaining the metabolic oxidation–reduction reaction and other less-potent ROS, such as hydrogen peroxide and nitric oxide. The first feature is especially favorable in inner-ear medicine, because many therapeutic compounds are blocked by the blood–cochlear barrier and cannot reach cochlear hair cells [17,18]. Therefore, hydrogen therapy could be widely used in medical applications as a safe and effective antioxidant with minimal side effects.

Conclusion

In conclusion, this study showed that hydrogen gas markedly decreased oxidative stress by scavenging ROS, and protected cochlear cells and tissues against oxidative stress. These results have prompted us to perform in-vivo studies to determine whether treatment with hydrogen gas might exert a beneficial effect on damaged cochleae and promote hearing recovery.

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OTOLOGY

Efficiency of a transtympanic approach to the round window membrane using a microendoscope

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Abstract There has been increasing interest in cochlear drug delivery through the round window membrane (RWM). However, placing drugs on the RWM is difficult because of anatomical barriers. We examined the efficacy of a microendoscope for a transtympanic approach to the RWM. We evaluated the visibility of the RWM using four approaches: transtympanic microendoscopic, transtympanic microscopic, transmastoid microendoscopic, and transmastoid microscopic in ten human temporal bones. For the transtympanic approach, we made a fenestration (2 × 1 mm) in the postero-inferior quadrant of the tympanic membrane. For the transmastoid approach, conventional posterior hypotympanotomy was performed. The transtympanic microendoscopic approach enabled visualization of the RWM in all specimens, whereas the transtympanic microscopic approach only permitted visualization in three specimens. Through the transmastoid approach, the RWM was visible in all specimens using either a microendoscope or a microscope. The transtympanic microendoscopic approach can be utilized for cochlear drug delivery through the RWM.

Keywords Microendoscope · Round window membrane · Cochlea · Drug delivery

Introduction

Sensorineural hearing loss (SNHL) is one of the most common disabilities in industrial countries. Systemic administration of steroids has been widely used for the treatment of acute profound hearing loss [1]; however there are limitations in their clinical efficacy [2]. At present, therapeutic strategies are limited to hearing aids and cochlear implants for patients with chronic SNHL. Based on this background, basic investigations have elucidated several agents that are effective for the treatment of SNHL. However, the problem of how to deliver drugs to the inner ear has been a considerable obstacle to the development of treatments for SNHL. The blood-inner ear barrier prevents the transportation of serum drugs to the inner ear, and the blood flow to the inner ear is very limited.

Drug transduction through the round window membrane (RWM) is one option for delivering drugs into the inner ear. Continuous infusion of RWM with an osmotic pump and microcatheter has been reported as an effective and safe approach [3]. However, it requires surgery and the invasion cannot be overlooked. Recently, new local drug application procedures using biodegradable substances are gaining interest [4, 5]. The inner ear is one of the targets for local drug administration using biodegradable gelatin hydrogels [6, 7]. In this drug delivery system, positively charged proteins or peptides are electrostatically trapped in negatively charged gelatin polymer chains. As the gelatin polymer chains degrade, proteins or peptides are released from the hydrogel. The released protein is conveyed through the RWM into the inner ear via a concentration gradient. Therefore, close contact of biodegradable hydrogels with the RWM is critical for efficient drug delivery to inner ear fluids.

The RWM is situated perpendicular to the tympanic membrane and deep in the round window niche. In some cases, a false membrane covers the RWM. For safe and certain drug administration, hydrogels containing drugs should be placed on the RWM under direct visualization. Use of a

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