produced in the cochlea (Kawata et al., 1988) and that all EP subtypes are expressed in various types of cochlear cell (Hori et al., 2009, 2010; Stjernschantz et al., 2004), suggesting that EP signaling might play roles in the physiology and pathophysiology of the cochlea. The present study focused on EP4 because of its reported protective effects on neurons against neuronal degeneration in various contexts (Ahmad et al., 2005; Esaki et al., 2010; Li et al., 2008; Shi et al., 2010; Umemura et al., 2010).

In the current study, we evaluated the functionality and morphology of cochleae in EP4-deficient mice under physiological conditions or following noise exposure. Pharmacological inhibition or activation of EP4 signaling was examined using mouse models of noise-induced hearing loss in order to confirm the findings.

2. Materials and methods

2.1. Animals and reagents

EP4-deficient mice were generated as described previously (Segi et al., 1998). Most EP4-deficient mice die postnatally as a result of patent ductus arteriosus and do not survive on a C57BL/6 background (Nguyen et al., 1997; Segi et al., 1998). Male EP4-deficient mice with a mixed 129/Ola and C57BL/6 genetic background (Kabashima et al., 2002) aged 8 weeks were used. Male mice with a similar mixed genetic background to the EP4-deficient mice were used as wild-type animals. The pharmacological inhibition and enhancement of EP4 signaling were investigated using 8-week-old male C57BL/6 mice (Japan SLC, Inc., Hamamatsu, Japan).

Animals were maintained at the Institute of Laboratory Animals, Kyoto University, Japan, under a 12-h light/12-h dark cycle and specific pathogen-free conditions. Mice were fed *ad libitum* with standard chow. Bedding and water bottles were replaced daily. The Animal Research Committee of the Graduate School of Medicine, Kyoto University, approved all experimental protocols.

The EP4 antagonist ONO-AE3-208 and the EP4 agonist ONO-AE1-329 were supplied by Ono Pharmaceutical, Co., Ltd (Osaka, Japan). Rabbit anti-βIII tubulin antibody was purchased from Covance Research Products (Berkeley, CA). Rabbit anti-myosin VIIa polyclonal antibody was purchased from Proteus BioSciences (Ramona, CA). Alexa 488 or 568-conjugated goat anti-rabbit antibody, DAPI, and fluorescein–phalloidin were purchased from Molecular Probes (Eugene, OR). Rabbit anti-EP4 receptor polyclonal antibody was purchased from Caymann Chemical (Ann Arbor, MI).

2.2. Auditory brainstem response (ABR)

Measurements of ABRs have been performed as described previously (Kada et al., 2009). Thresholds were determined for frequencies of 10, 20, and 40 kHz from a set of responses at varying intensities with 5-dB sound pressure level (SPL) intervals. When no response was present at the highest sound level available, the threshold was designated as being 5 dB greater than that level for statistical purposes. The thresholds at each frequency were verified at least twice.

2.3. Distortion-product otoacoustic emissions (DPOAEs)

Recordings were made with an acoustic probe (ER-10C; Etymotic Research, Elk Grove Village, IL) using the DP2000 DPOAE measurement system version 3.0 (Starkey Laboratory, Eden Prairie, MN). Two primary tones with an f2/f1 ratio of 1.2 were presented at intensity levels of 65 dB SPL (L1) and 55 dB SPL (L2). The f2 was varied in one-ninth-octave steps from 8 to 16 kHz. A peak at 2f1–f2 in the spectrum was recognized as a DPOAE. The DP/noise floor (NF) levels were calculated and statistical analyses were performed at each f2 frequency.

2.4. Endocochlear potential

Measurements of the endocochlear potential were performed as described previously (Kada et al., 2009). A silver–silver chloride reference electrode was placed under the skin of the dorsum. A micropipette electrode (10–40 MO) filled with 150 mM KCl was advanced through the bony aperture into the spiral ligament. The entry of the electrode tip into the endolymph was characterized by fast changes of the recorded potentials. The electrode was advanced until a stable potential was observed, at which point no alterations were dependent upon its depth. The signal was passed through an amplifier (Duo 773; World Precision Instruments, Sarasota, FL).

2.5. Surgical procedure for topical application

The EP4 antagonist ONO-AE3-208 was dissolved in 1 N NaOH and diluted with PBS to give a final concentration of 1 mg/ml and a final pH of 7.4. The EP4 agonist ONO-AE1-329 was dissolved in DMSO and diluted with physiological saline to give a final concentration of 1 mg/ml containing 1% dimethyl sulfoxide (DMSO). Both drugs were applied topically under general anesthesia. The otic bulla of the left temporal bone was exposed via a retroauricular approach. A small hole was made in the otic bulla to access the round window membrane (RWM). A dry gelatin sponge was cut into 0.5–1 mm^3 pieces and a piece of the sponge placed on the RWM following the immersion of substrates (2 μl). Control animals received topical application of PBS at pH 7.4 or physiological saline containing 1% DMSO.

2.6. Noise exposure

Animals under general anesthesia were placed in a ventilated sound-exposure chamber fitted with speakers driven by a noise generator and a power amplifier. A 1/2-inch condenser microphone and a fast Fourier transform analyzer (both from Sony, Tokyo, Japan) were used to monitor and calibrate sound levels at multiple locations within the chamber, in order to ensure uniformity of the stimulus. The stimulus intensity varied by a maximum of 3 dB SPL across measured sites within the exposure chamber.

2.7. Histology

After the functional analyses, the cochleae were subjected to histological analysis as whole mounts or 10-μm-thick frozen sections. The whole-mount samples were used for the histological evaluation of hair cells. The frozen sections were used for the evaluation of gross anatomy by hematoxylin and eosin (H&E) staining, for immunohistochemistry for EP4 or for quantitative assessments of spiral ganglion neurons.

The whole-mount specimens were separated into three regions with distances from the apex of 20–40% (corresponding to 8–16 kHz regions; apical portion), 40–70% (corresponding to 16–32 kHz regions; mid-basal portion), and 70–90% (corresponding to 32–64 kHz regions; basal portion) (Viberg and Canlon, 2004). After permeabilization with 0.2% Triton X in PBS for 30 min at room temperature, the specimens were incubated with anti-myosin VIIa rabbit polyclonal antibody (1:500) for 12 h at 4 °C, followed by incubation with Alexa-568 conjugated anti-rabbit goat IgG (1:500) secondary antibody. At the end of the staining procedures, the specimens were stained with fluorescein–phalloidin (1:400) and 4′,6-diamino-2-phenylindole (DAPI) for 15 min at room temperature, and viewed with a confocal microscope (TCS SPE; Leica Microsystems, Wetzlar, Germany). The respective numbers of inner hair cells (IHCs) and outer hair cells (OHCs) were counted, and the ratio of missing IHCs and OHCs was calculated for each region of cochleae.

Two mid-modiolar sections (separated by a distance of 40–50 μm) from each cochlea were used for immunostaining for EP4 or for the histological analysis of spiral ganglion neurons, respectively. Immunohistochemistry for EP4 was performed with anti-EP4 receptor (1:200) primary antibody and Alexa 568-conjugated anti-rabbit goat IgG (1:500) secondary antibody. Immunohistochemistry for βIII-tubulin was performed to identify the spiral ganglion neurons in the Rosenthal's canal. Specimens were treated with rabbit anti-βIII tubulin (1:250) primary antibody and Alexa-488 conjugated anti-rabbit goat IgG (1:500) secondary antibody, followed by nuclear labeling with DAPI. The specimens were then observed using a fluorescence microscope (Olympus BX50, Tokyo, Japan). The numbers of spiral ganglion neurons and the area of the Rosenthal's canal were quantified, and the density of spiral ganglion neurons was calculated as described previously (Kada et al., 2009).

2.8. Statistical analysis

Data are expressed as the mean ± SEM for the indicated number of observations. The unpaired Student's *t*-test (two-tailed) was used, as appropriate, for comparisons between two groups. Two-way factorial analysis of variance (ANOVA) was used for comparisons of ABR-threshold shifts after noise exposure, and the Tukey–Kramer test was performed for pair-wise comparisons. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Hearing loss in EP4-deficient mice

ABR recordings for screening the auditory function of EP4-deficient mice demonstrated significant but slight hearing loss at frequencies of 10, 20, and 40 kHz. EP4-deficient mice ($n = 16$) exhibited significant elevation of ABR thresholds in comparison with wild-type mice ($n = 16$) at 10 kHz ($P < 0.0001$), 20 kHz ($P < 0.0001$), and 40 kHz ($P < 0.0001$) (Fig. 1A). To examine the mechanisms underlying the hearing impairment in EP4-deficient mice, we measured DPOAEs, which reflect the OHCs in the cochlea (Parham et al., 1999), and the endocochlear potential, which is the positive voltage in the endolymphatic space of the

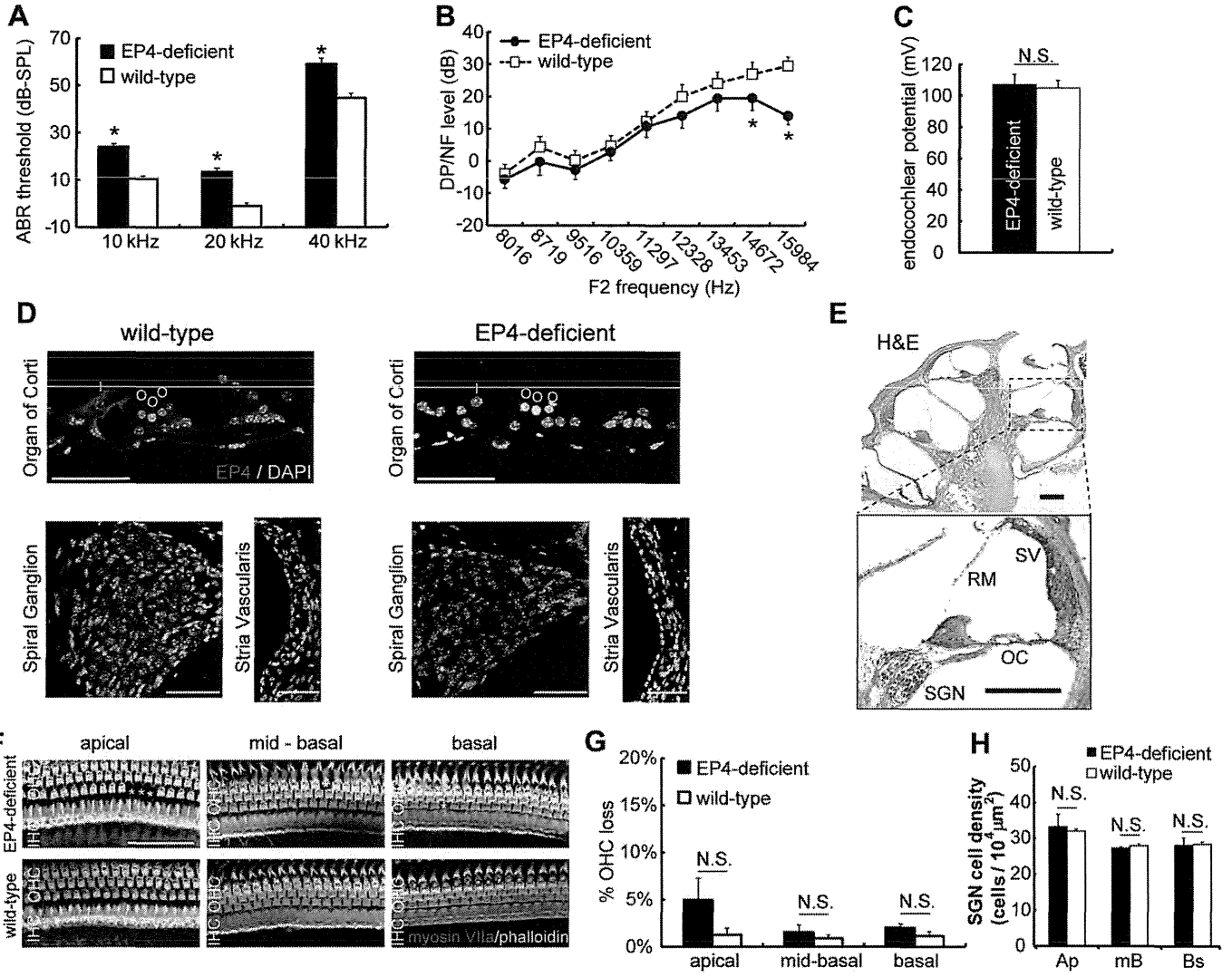

Fig. 1. Auditory function and cochlear morphology of EP4-deficient mice. (A) The ABR thresholds in EP4-deficient mice differed significantly at each frequency in comparison with those in wild-type mice. Asterisk indicates a significant difference ($P < 0.05$). (B) The DP/NF levels of EP4-deficient mice were significantly lower than those of wild-type mice at f2 frequencies of 15,984 and 14,672 Hz. Asterisk indicates a significant difference ($P < 0.05$). (C) No statistical difference in the endocochlear potential was found between EP4-deficient and wild-type mice. (D) Immunostaining for EP4 (red) and nuclear staining with DAPI (blue) showed EP4 expression in an inner hair cell (I) and outer hair cells (O) of the organ of Corti, spiral ganglion neurons and stria vascularis in a wild-type cochlea, while no expression was found in an EP4-deficient cochlea. Scale bar, 50 μm. (E) H&E staining of a cross section of an EP4-deficient cochlea revealed no abnormalities. OC, organ of Corti; RM, Reissner's membrane; SGN, spiral ganglion neuron; SV, stria vascularis. Scale bars, 200 μm. (F) Immunostaining for myosin VIIa (red) and F-actin labeling with phalloidin (green) revealed loss of OHCs in EP4-deficient mice (asterisked). Scale bar, 50 μm. (G) The percentages of OHC loss in each part of cochleae in EP4-deficient and wild-type mice are shown. (H) No significant differences in the densities of spiral ganglion neuron (SGN) were found in the apical (Ap), mid-basal (mB) or basal (Bs) portions of cochleae between EP4-deficient and wild-type mice. In all graphs, the error bars represent the SEM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

cochlea that is mainly generated by the stria vascularis (Tasaki and Spyropoulos, 1959).

In DPOAE measurements, EP4-deficient animals demonstrated significant loss in the DP/NF levels at f2 frequencies of 14,672 ($P < 0.0001$) and 15,984 Hz ($P = 0.0255$) compared with wild-type animals ($n = 16$ for each; Fig. 1B). By contrast, the mean endocochlear potential of the EP4-deficient mice (107.3 ± 6.5 mV) did not differ significantly from that of wild-type mice (104.8 ± 5.1 mV; n=4 for each; Fig. 1C). These functional assessments suggested that the EP4-deficient mice had slight hearing loss due to OHC dysfunction.

To investigate the morphological phenotypes of the EP4-deficient mice, cochlear specimens were examined using either cryostat sections or surface preparations. The expression of EP4 was found in in the stria vascularis, spiral ganglion neurons, supporting cells, IHCs and OHCs in wild-type mice (Fig. 1D) similarly to our previous observation (Hori et al., 2009), while no expression of EP4 was identified in EP4-deficient mice (Fig. 1D). H&E staining of cross sections revealed that the gross anatomy of EP4-deficient mice cochleae was essentially normal (Fig. 1E). No apparent degeneration was seen in the organ of Corti, spiral ganglion, or cochlear lateral wall including the stria vascularis at the light-microscopic level. Immunostaining for myosin VIIa and f-actin labeling with phalloidin revealed the surface morphology of the organ of Corti. Loss of OHCs was observed in the basal, mid-basal, and apical portions of EP4-deficient cochleae (Fig. 1F). OHC loss was confirmed by the nuclear staining with DAPI. However, there was no statistically significant difference in OHC loss between EP4-deficient and wild-type mice (Fig. 1G). This finding suggested an involvement of other mechanisms besides OHC degeneration for hearing loss in EP4-deficient mice. We then quantitatively analyzed the density of

spiral ganglion neurons, of which loss causes ABR-threshold shifts, using immunostaining for βIII tubulin in cross sections, which showed no significant loss of spiral ganglion neurons (Fig. 1H). We therefore considered that EP4 deficiency affected auditory systems including OHC function, which might result in modest hearing loss under physiological conditions.

3.2. Vulnerability to noise trauma in EP4-deficient mice

Mouse models of noise-induced hearing loss have previously been used to test the roles of various molecules in pathophysiological conditions of the auditory system (Peppi et al., 2011; Polesskaya et al., 2010; Tan et al., 2010). Noise-induced damage sometimes clarified roles of molecules in auditory function. We therefore examined the effects of EP4 deficiency on noise-induced hearing loss, in order to clarify its role in auditory function. EP4-deficient and wild-type mice ($n = 12$ for each) were exposed to an octave-band noise centered on 8 kHz at a 120-dB SPL for 2 h, and then subjected to ABR measurements 7 and 14 days later. Alterations in the ABR-threshold shifts of EP4-deficient and wild-type mice are shown in Fig. 2A. The overall effects of EP4 deficiency on the ABR-threshold shifts were statistically significant at 10 kHz (df = 1, $F = 5.287$ and $P = 0.0247$), 20 kHz (df = 1, $F = 10.720$ and $P = 0.0017$), and 40 kHz (df = 1, $F = 17.323$ and $P < 0.0001$) with two-way factorial ANOVA. Pair-wise comparisons with Tukey–Kramer test revealed significantly higher elevations of ABR thresholds in EP4-deficient than in wild-type mice on day 14 at 20 kHz, and on days 7 and 14 at 40 kHz. DPOAE assessments demonstrated significant decreases of DP/NF levels at 11,297 Hz ($P = 0.0005$), 13,453 Hz ($P = 0.0068$), 14,672 Hz ($P = 0.0371$), and 15,984 Hz ($P = 0.0006$) in EP4-deficient mice compared with wild-type mice (Fig. 2B). These findings demonstrated that noise exposure revealed auditory system differences between EP4-deficient and wild-type mice, in particular high frequency regions. By contrast, there was no significant difference in the endocochlear potential between EP4-deficient (103.3 ± 2.5 mV) and wild-type (97.3 ± 3.5 mV) mice (Fig. 2C).

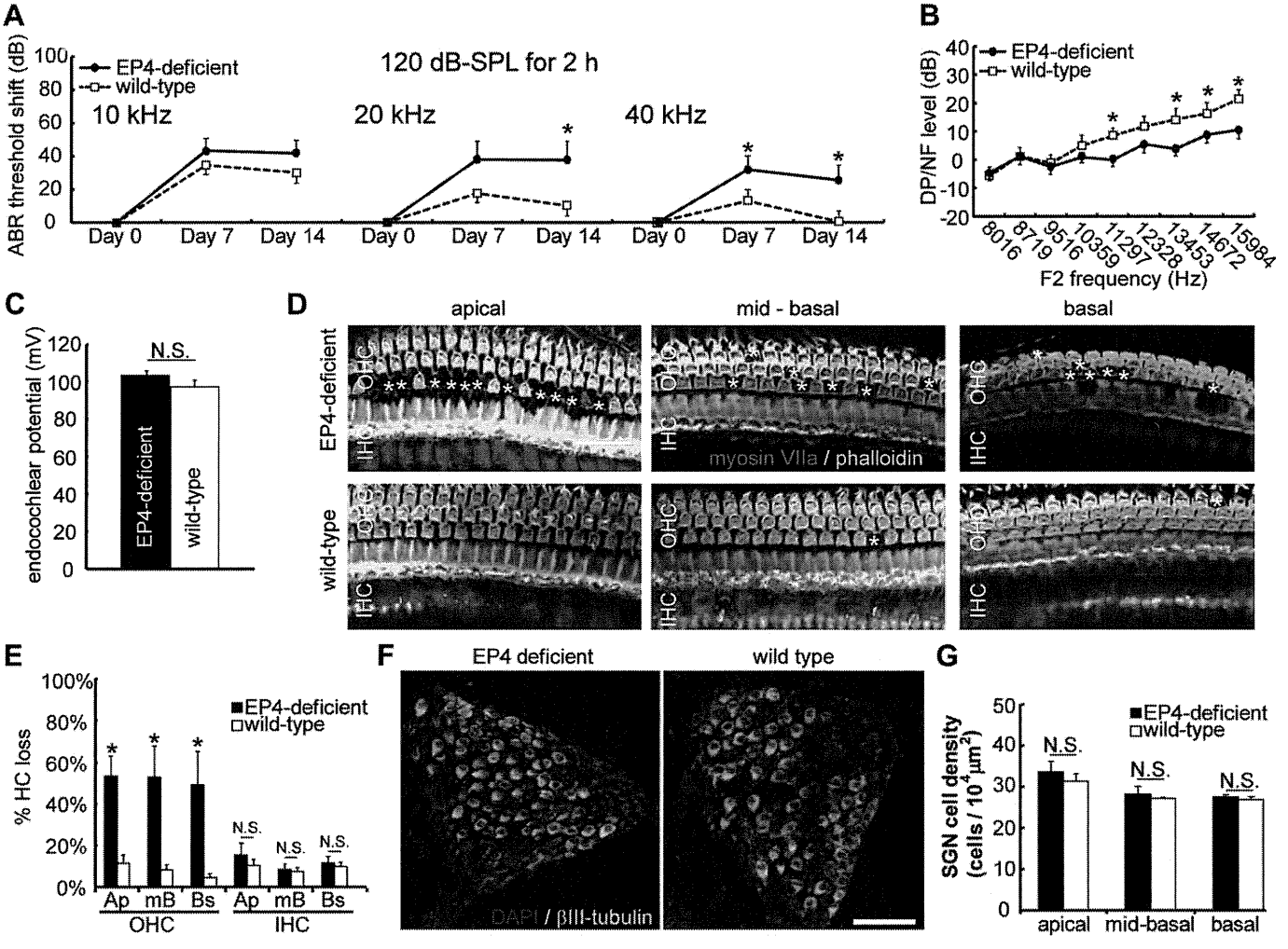

Fig. 2. Vulnerability of EP4-deficient mice to noise trauma (an octave-band noise centered on 8 kHz at a 120-dB SPL for 2 h). (A) The time courses of the alterations in the ABR-threshold shifts of EP4-deficient and wild-type mice at frequencies of 10, 20, and 40 kHz are shown. The overall effects of EP4 deficiency on the ABR-threshold shifts were statistically significant at all frequencies. Asterisks indicate significant differences in ABR-threshold shifts in pair-wise comparisons. (B) Significant decreases in DP/NF levels in EP4-deficient mice were observed at 11,297, 13,453, 14,672 and 15,984 Hz. Asterisk indicates a significant difference ($P < 0.05$). (C) The endocochlear potential in EP4-deficient mice was similar to that in wild-type mice after noise trauma. (D) Immunostaining for myosin VIIa (red) and F-actin labeling with phalloidin (green) revealed extensive loss of OHCs in each cochlear portion of EP4-deficient mice (asterisked). Scale bar, 20 μm. (E) EP4-deficient mice showed significantly higher levels of OHC loss in the apical (Ap), mid-basal (mB), and basal (Bs) portions of cochleae compared with wild-type mice. Asterisk indicates a significant difference ($P < 0.05$). (F) Immunostaining for βIII-tubulin (green) and nuclear staining for DAPI (blue) revealed the spiral ganglion neurons of EP4-deficient mice to be normal. Scale bar, 50 μm. (G) There was no significant difference in the density of spiral ganglion neurons (SGN) between EP4-deficient and wild-type mice. In all graphs, the error bars represent the SEM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Morphological analyses confirmed that EP4 deficiency enhanced the degeneration of OHCs in response to noise. Cochlear specimens were obtained 14 days after noise exposure. Noise-induced damage to the organ of Corti was assessed by immunostaining for myosin VIIa and f-actin labeling with phalloidin in whole-mount preparations. Minor loss of OHCs was observed in the basal, mid-basal, and apical portions of wild-type cochleae, whereas the EP4-deficient mice exhibited extensive loss of OHCs (Fig. 2D). A quantitative analysis demonstrated significant decreases of OHCs in the apical ($P = 0.0058$), mid-basal ($P = 0.0330$), and basal ($P = 0.0400$) portions of the cochleae of EP4-deficient mice in comparison with those of wild-type mice (Fig. 2E). No significant difference in inner hair cells (IHCs) was found between EP4-deficient and wild-type mice (Fig. 2E). Immunostaining for βIII tubulin in cross sections revealed no degeneration of spiral ganglion neurons in either EP4-deficient or wild-type mice (Fig. 2F), and quantitative assessments revealed no significant difference in the density of spiral ganglion neurons between EP4-deficient and wild-type mice ($n = 4$ for each; Fig. 2G). Consequently, morphological differences between EP4-deficient and wild-type mice also appeared to be apparent following noise exposure, which suggests that the OHC is included in targets of cochlear damage due to EP4 deficiency. These findings demonstrate that EP4-deficient mice are vulnerable to noise-induced damage in comparison with wild-type animals, indicating the importance of EP4 for cochlear protection, particularly in OHCs, against noise trauma.

3.3. Inhibition of EP4 signaling enhanced noise-induced cochlear damage

The pharmacological inhibition of EP4 signaling was investigated in order to confirm the effects of EP4 deficiency on noise-induced hearing loss. The EP4 antagonist ONO-AE3-208 (Kabashima et al., 2002; Sugimoto and Narumiya, 2007) was topically applied to the RWM in the middle-ear cavity of 8-week-old C57BL/6 mice. Immediately after the topical application, animals were exposed to an octave-band noise centered on 8 kHz at 120 dB SPL for 1 h. Both control and EP4 antagonist-treated animals ($n = 5$ for each) showed similar elevations of ABR thresholds on day 1 after noise exposure (Fig. 3A), indicating that both groups experienced similar levels of initial damage. However, the subsequent recovery process differed notably between the two groups (Fig. 3A). In control animals, the ABR-threshold shifts showed a trend toward recovery at all frequencies, whereas no recovery was observed in EP4 antagonist-treated animals (Fig. 3A). Statistical analyses revealed significant differences in the ABR-threshold shifts between the two groups at each frequency. The overall effects of EP4 antagonist application were significant at 10 kHz ($df = 1$, $F = 10.195$ and $P = 0.0032$), 20 kHz ($df = 1$, $F = 10.662$ and $P = 0.0026$), and 40 kHz ($df = 1$, $F = 15.703$ and $P = 0.0004$). Pair-wise comparisons revealed significant differences in the ABR-threshold shifts on day 7 at 10 and 20 kHz, and on days 7 and 14 at 40 kHz. These findings demonstrate that pharmacological inhibition of EP4 had a similar effect to genetic deletion in enhancing noise-induced hearing loss.

Morphological assessment demonstrated the enhancement of noise-induced damage to OHCs caused by pharmacological inhibition of EP4 signaling. Immunostaining for myosin VIIa and f-actin labeling with phalloidin in whole-mount preparations revealed an enhancement of OHC loss in the basal, mid-basal, and apical portions of cochleae by the EP4 antagonist (Fig. 3B). A quantitative analysis demonstrated significantly greater OHC loss in the basal ($P = 0.0473$), mid-basal ($P = 0.0089$), and apical ($P = 0.0282$) portions of cochleae treated with the EP4 antagonist (Fig. 3C). By contrast, no significant difference in IHC loss was found between the two groups (Fig. 3C). These findings revealed that

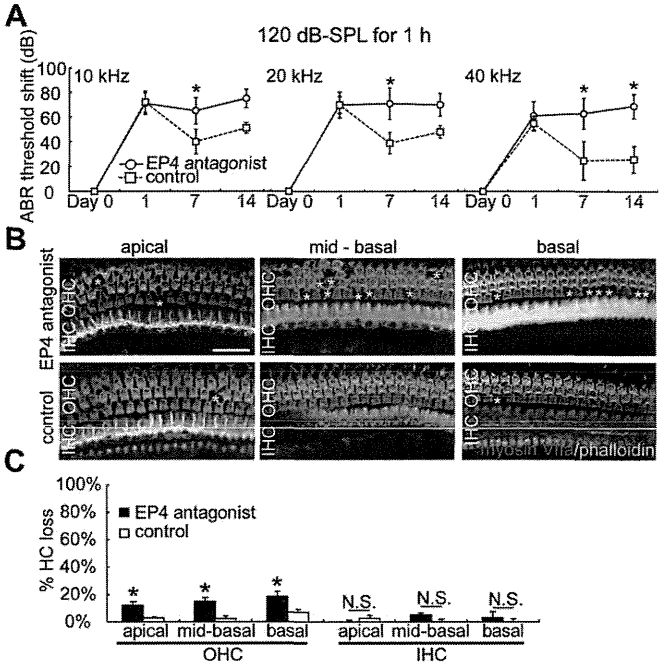

Fig. 3. Pharmacological inhibition of EP4 accelerated noise-induced damage in cochleae. The EP4 antagonist ONO-AE3-208 was topically applied to the RWM and control mice received a topical application of PBS at pH 7.4. (A) The time courses of the alterations in the ABR-threshold shifts of EP4 antagonist-treated and control mice at frequencies of 10, 20, and 40 kHz are shown. The overall effects of EP4 antagonist application were significant at all frequencies. Asterisks indicate significant differences in ABR-threshold shifts between EP4 antagonist-treated and control mice with pair-wise comparisons. (B) Immunostaining for myosin VIIa (red) and F-actin labeling with phalloidin (green) demonstrated extensive loss of OHCs in the basal, mid-basal, and apical portions of cochleae treated with the EP4 antagonist. Scale bar, 20 μm. (C) A quantitative analysis demonstrated significant differences between the two groups in the numbers of lost OHCs in the apical, mid-basal, and basal portions of cochleae. Asterisk indicates a significant difference ($P < 0.05$). No significant difference in IHC numbers was found between the two groups. In all graphs, the error bars represent the SEM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

pharmacological inhibition of EP4 had a similar effect to genetic deletion in enhancing OHC degeneration due to noise exposure. EP4 signaling might therefore play a role in protecting OHCs against noise-induced damage.

3.4. EP4 agonist attenuated noise-induced cochlear damage

Both genetic deletion and pharmacological inhibition of EP4 enhanced noise-induced damage to cochleae. These findings suggested that the activation of EP4 signaling could attenuate noise-induced hearing loss. We tested the effects of the EP4 agonist ONO-AE1-329 (Sugimoto and Narumiya, 2007; Suzawa et al., 2000) on noise-induced damage in 8-week-old C57BL/6 mice. Immediately after drug application, the animals were exposed to an octave-band noise centered on 8 kHz at 120 dB SPL for 2 h. Control animals ($n = 5$) showed ABR-threshold shift of approximately 80 dB on day 7 and no recovery on day 14. The animals treated with the EP4 agonist ($n = 5$) exhibited comparatively small ABR-threshold shifts on day 7, and showed a trend for decreasing shifts until day 14 (Fig. 4A). Statistical analyses revealed that local application of the EP4 agonist had significant effects on ABR-threshold shifts at 10 kHz ($df = 1$, $F = 25.000$ and $P < 0.0001$), 20 kHz ($df = 1$, $F = 9.164$ and $P = 0.0058$), and 40 kHz ($df = 1$, $F = 36.152$ and $P < 0.0001$). Pair-wise comparisons showed significant differences between the two groups in ABR-threshold shifts on days 7 and 14 at 10 kHz, on

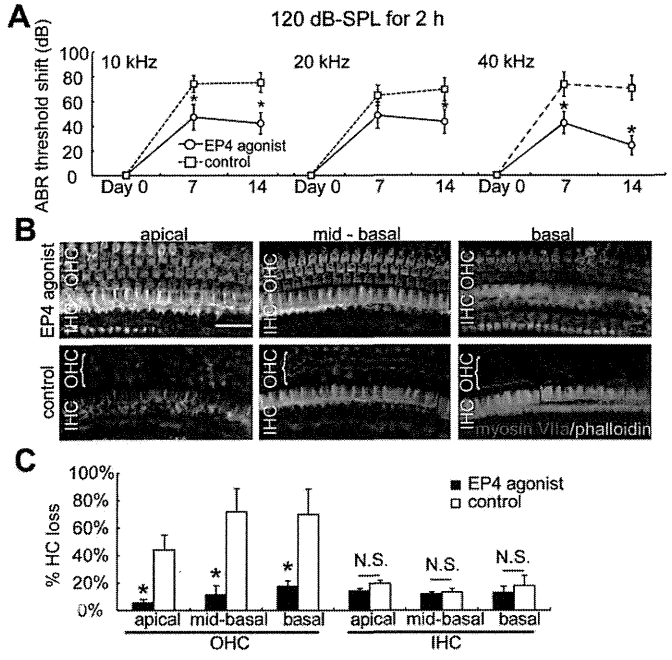

Fig. 4. The EP4 agonist ameliorated noise-induced damage to cochleae. The EP4 agonist ONO-AE1-329 was locally applied to the RWM and control animals received a local application of physiological saline containing 1% DMSO. (A) The time courses of the alterations in the ABR-threshold shifts of EP4 agonist-treated and control mice at frequencies of 10, 20, and 40 kHz are shown. The overall effects of local application of the EP4 agonist on ABR-threshold shifts were significant at all frequencies. Asterisks indicate significant differences in ABR-threshold shifts between EP4 agonist-treated and control mice with pair-wise comparisons. (B) Immunostaining for myosin VIIa (red) and F-actin labeling with phalloidin (green) showed severe loss of OHCs in control specimens, whereas the morphology of the organ of Corti was preserved in EP4 agonist-treated mice. Scale bar, 20 μm. (C) A quantitative analysis demonstrated significant differences between the two groups in the numbers of lost OHCs in the apical, mid-basal, and basal portions of cochleae. Asterisk indicates a significant difference ($P < 0.05$). No significant difference in IHC numbers was found between the two groups. In all graphs, the error bars represent the SEM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

day 14 at 20 kHz, and on days 7 and 14 at 40 kHz. These findings demonstrate that the topical application of an EP4 agonist attenuated noise-induced hearing loss.

Morphologically, severe degeneration of OHCs was observed from the apex to the base of cochleae in control animals (Fig. 4B). Approximately 70% of the OHCs in the mid-basal and basal portions of cochleae were destroyed by the noise exposure in control animals (Fig. 4C). In contrast to control animals, OHCs in specimens treated with the EP4 agonist were well maintained (Fig. 4B). Quantitative assessments showed that the EP4 agonist had a significant protective effect on OHCs in the apical ($P = 0.0209$), mid-basal ($P = 0.0215$), and basal ($P = 0.0455$) portions of cochleae (Fig. 4C). No significant difference in IHC loss was found between the two groups (Fig. 4C). These findings demonstrate that the pharmacological activation of EP4 signaling promoted the survival of OHCs exposed to noise trauma. These findings in pharmacological activation of EP4 support our hypothesis that EP4 signaling plays a crucial role in the maintenance of auditory function.

4. Discussion

The present study demonstrates that genetic deletion of EP4 causes slight elevation of ABR thresholds and loss of DPOAEs, although the endocochlear potential is maintained in normal levels. Genetic deletion of EP4 accelerated ABR-threshold shifts and loss of DPOAEs, but not of endocochlear potentials, following noise exposure. These findings in EP4-deficient mice indicate that EP4 may be involved in the maintenance of auditory function, in particular OHC function. In addition, pharmacological inhibition of EP4 signaling enhanced noise-induced hearing loss, and its pharmacological activation attenuated noise-induced hearing loss. Findings in pharmacological inhibition or activation support the findings in genetic deletion of EP4.

Experimental animals were exposed to an octave-band noise centered on 8 kHz at a 120-dB SPL, which causes cochlear damage not only in low frequency regions, but also middle and high frequency regions (Ou et al., 2000), for 1 or 2 h according to the experimental design. In experiments of genetic deletion or pharmacological inhibition of EP4, we intended to induce mild hearing loss in control animals, which is suitable for assessments of acceleration of noise-induced damage by toxic treatments. In genetic deletion of EP4, 2-h noise exposure was used, while 1-h exposure was used in pharmacological inhibition of EP4, because of the difference in back ground strains between two experiments. In genetic deletion of EP4, a mixed 129/Ola and C57BL/6 genetic background was used, while in pharmacological inhibition of EP4, we used C57BL/6 mice, which were reported to be more vulnerable to noise trauma than sub-strains of mouse strain 129 (Turner et al., 2005; Yoshida et al., 2000). We therefore used a shorter noise exposure period in pharmacological inhibition of EP4 than that in experiments using EP4-deficient mice. In experiments for pharmacological activation of EP4 signaling, we intended to generate profound hearing loss in control animals, which is suitable for assessments of protective effects. C57BL/6 mice were, therefore, exposed to an octave-band noise centered on 8 kHz at 120 dB SPL for 2 h, which was longer than that in pharmacological inhibition experiments. As we expected, profound hearing loss occurred in control animals, and significant protection by pharmacological activation of EP4 was observed.

The present study reveals that EP4 signaling plays a crucial role in the protection of OHCs against noise trauma. Extending the findings of our previous study that EP4 is expressed in the OHCs of the mouse cochlea (Hori et al., 2009), EP4 signaling could therefore act directly on OHCs. The activation of EP4 induces cyclic AMP production in OHCs, which might help to rescue them from energy depletion caused by overstimulation in response to excessive noise exposure. EP4 signaling has been reported to activate anti-apoptotic pathways associated with protein kinase A (Hoshino et al., 2003), phosphatidylinositol 3-kinase-mediated AKT phosphorylation (Liou et al., 2007), BCL-2 antagonist of cell death (BAD) (Chun et al., 2007) and survivin (Baratelli et al., 2005). Such mechanisms could be involved in OHC protection by EP4 signaling in mouse models of noise-induced hearing loss.

The expression of EP4 was also identified in a variety of cell types in the mouse cochlea (Hori et al., 2009). Therefore, indirect effects of EP4 signaling could contribute to the promotion of the survival of OHCs. Previously, pharmacological activation of EP4 has been reported to induce generation of vascular endothelial growth factor in spiral ganglion neurons (Hori et al., 2010), which may contribute to the survival of OHCs against noise-induced damage (Picciotti et al., 2006; Selivanova et al., 2007).

There are some discrepancies between the functional and morphological findings in the present study. In EP4-deficient mice under a physiological condition, slight ABR-threshold shifts were found, whereas morphological analyses demonstrated essentially normal morphology of cochleae except for limited loss of OHCs. C57/BL6 mice treated with an EP4 antagonist exhibited slight loss of OHCs, despite of remarkable ABR-threshold shifts following noise exposure. These findings suggest that other targets of EP4 signaling in the cochlea besides OHCs may play a role in hearing loss due to

EP4 deficiency. EP4 expression was also found in IHCs and spiral ganglion neurons, which are also involved in mechanisms for noise-induced hearing loss (Pujol and Puel, 1999). Hence, the IHC and spiral ganglion neuron could contribute to hearing loss due to genetic deletion or pharmacological inhibition of EP4. However, no significant loss of IHCs or spiral ganglion neurons was found in EP4-deficient mice under a physiological condition or after noise trauma in the present study. Therefore, degeneration at the substructural level might occur in IHCs and spiral ganglion neurons, in particular in afferent dendrites attached to the base of the IHC, which is known as a target of noise trauma (Pujol and Puel, 1999). Future studies using electron microscopy are required to elucidate detailed mechanisms underlying hearing loss due to lack of EP4 signaling.

In conclusion, the present findings demonstrate an involvement of EP4 signaling in the maintenance of the auditory system, and of OHCs in particular. The roles of other PGE receptors, including EP1–3, in the cochlea should also be determined, in order to understand the roles of PGE_2 signaling in the auditory system. This could help to identify new targets for cochlear disease therapeutics.

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JOHNS
特集●急性感音難聴の最新治療戦略●

急性感音難聴における新規治療の可能性

ナノDDS

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● Key Words ●急性感音難聴，ドラッグ・デリバリー・システム，ナノテクノロジー，内耳●

I．“ナノ”とは？

ナノとは，国際単位系に用いられる用語で，基礎となる単位の10^{-9}倍であることを意味する，といわれても，小さいということ以外は，ピンと来ないと思われる。長さの単位メートルで具体的に説明すると，1メートルが概ねヒトの身長のサイズを示すとすると，髪の毛がミリ（10^{-3}）メートルの単位となる。次がマイクロ（10^{-6}）メートルで，組織学でよく出てくる単位で，細胞やミトコンドリアの大きさの単位である。ナノ(10^{-9})メートルは，細胞を構成しているタンパクや遺伝子のサイズになる。

ウイルスの大きさもナノメートル単位である。つまり，ナノパーティクルとは，ナノメートルサイズの粒子ということを表し，細胞よりもはるかに小さいサイズの粒子を使った薬物送達システムの内耳への応用が本稿の要旨となる。ちなみに，ナノテクノロジーとは，ナノメートルサイズでの加工技術であり，それにより製作される機械はナノマシンと呼ばれる。ちなみに，ナノの次に小さい10^{-12}単位は，ピコである。ナノパーティクルは，われわれの生活のさまざまな分野ですでに活用されている。例えば，トイレの脱臭触媒に金属系のナノパーティクルが用いられている。

II．“DDS”とは？

ドラッグ・デリバリー・システムの略であることは，ご存じであろう。直訳的に説明を加えれば，より良い効果を獲得するために薬物を送達するシステムとなる。もう少し具体的に表現すると，薬物をマテリアルで修飾することによって薬物の作用を高める工夫，方法となる。では，どのようにして，薬物の効果を高めるのかというと，

1）薬物の徐放
2）薬物の体内半減期の延長
3）薬物の透過，吸収の促進
4）薬物を目的細胞のみに取り込ませる（ターゲッティング）

などを目的として，薬物のマテリアルによる修飾がなされる。

ナノDDSは，ナノスケールの粒子の中に薬物を封入するDDS技術であり，上記の1）～4）のすべての目的に用いることができる。外殻のマテリアルの生体内での溶け方により，徐放がコントロールできる。パーティクルの表面加工により，肝臓など細網系への取り込みを逃れることができる。表面に特殊な修飾を行うことによって，標的細胞にのみ親和性を持たせるといった加工がなされている。

ナノスケールであることの最大の特徴は，全身投与に使えるという点と細胞内にパーティクルとして取り込まれるという2点に集約される。細胞内に粒子として到達することが可能であることから，遺伝子導入にも応用できる。また，サイズを調節することにより，正常の毛細血管からは漏れないが，癌組織の毛細血管は透過するように調節可能であり，癌細胞への受動的ターゲッティングがなされている[1]。この技術は，enhanced permeation and retention（EPR）効果としてよく知られており[2]，すでに臨床で用いられている抗癌剤（ドキシル®）に使われている。粒子の材料となる

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マテリアルは数多くあり，さまざまな使途がある。興味のある方は，成書をご参照いただきたい[3]。

Ⅲ．感音難聴治療におけるDDSの重要性

感音難聴の薬物治療における主な標的は，蝸牛である。蝸牛に存在する有毛細胞，ラセン神経節細胞，血管条やラセン靭帯の細胞が標的細胞となる。蝸牛は，血流量が少なく，血液内耳関門があるために，全身投与された薬物は，蝸牛内の細胞に到達しにくい[4]。十分な効果を発揮させるためには，大量・長期投与が必要となり，副作用のリスクは高くなる。全身投与では，なんらかのDDS技術を使わなければ，蝸牛への適切な薬物移行は実現できない。

蝸牛への薬物投与方法としては，経正円窓膜投与を中心とした局所投与がある。正円窓膜を通過する薬物であれば，全身的な副作用のリスクを大きく軽減しつつ，多くの薬物が蝸牛内に移行することが期待できる。しかし，鼓室内に投与された薬物は直ちに耳管から排泄されてしまうし，単純な鼓室内注入では，ごく限られた時間，蝸牛の基底部に薬物が到達するのみであり，局所投与においてもDDSの応用が必要となる[4]。

DDSの内耳への応用としては，2つの流れがある。ひとつは，既存の臨床で使用可能である薬物をDDSを用いることによって，内耳治療に使えるようにしようとする工夫である。われわれが行っているインスリン様細胞増殖因子1（IGF1）をゼラチンハイドロゲルというDDSを用いて内耳治療に応用する研究[5]が，これに該当する。この方法は，局所投与への応用であり，動物実験[6]から開始し，現在は第Ⅱ相臨床試験を行っている段階にある。もうひとつは，これまで感音難聴治療に使われてきた薬物をDDS技術の応用により効果を高める研究である。局所投与，全身投与，両方で研究が行われているが，最も広く行われている研究は，ステロイド局所投与におけるDDS応用である[7]。

Ⅳ．内耳領域におけるナノDDSの応用

内耳におけるナノDDS研究は，薬物送達と遺伝子導入という2つの方向で研究が行われている。われわれも早くからナノパーティクルの応用に着目しており，シンプルなポリ乳酸およびポリグリコールを材料とするナノパーティクルの内耳DDSへの応用に関する研究を行った[8]。

前述したようにナノパーティクルは，全身投与にも用いることができるので，第一に全身投与した場合の蛍光色素を含有するナノパーティクルの蝸牛での分布を肝臓や腎臓と比較した。蝸牛の血管系にナノパーティクルの局在を認めたが，有意なものではなかった[8]。実際，ステロイドを含有するナノパーティクルを用いて，音響外傷に対する効果を調べたが，通常のステロイドよりも優れた効果は認められなかった。

シンプルなポリ乳酸およびポリグリコールを材料とするナノパーティクルが有効でなかった原因として，多くのナノパーティクルが全身投与後，肝臓などの細網系でとらえられてしまい徐放効果が得られにくいことがわかった。そこで，ナノパーティクルの表面をポリエチレングリコールで修飾し，肝臓などの細網系でとらえられない工夫を行った[9]。すると，通常のステロイドを投与した場合と比較して，有意に多い量のステロイドが長期にわたって蝸牛に到達することが明らかとなった[9]。さらに，音響外傷に対する効果を調べたところ，通常のステロイドよりも有意に良好な聴覚改善効果が認められることがわかった[9]。したがって，ステロイドの全身投与において，ナノDDSは，急性感音難聴に対する治療効果を高める可能性があることが示唆されたといえる。

シンプルなポリ乳酸およびポリグリコールを材料とするナノパーティクルは，全身投与では有効なDDSではないことは前述したが，局所投与では有効性が期待できることが示されている[8]。正円窓膜上に留置されたナノパーティクルは，蝸牛内に移行し，鼓室階に分布することがわかった。すなわち，ナノパーティクルは正円窓膜を通過することが示されたこととなる。この結果は，他施設での実験でも同様の結果が示されており[10]，蝸牛内に薬物を運ぶキャリアーとしてナノパーティクルを用いることができることがわかった。この点は，マイクロパーティクルと大きく異なる点で

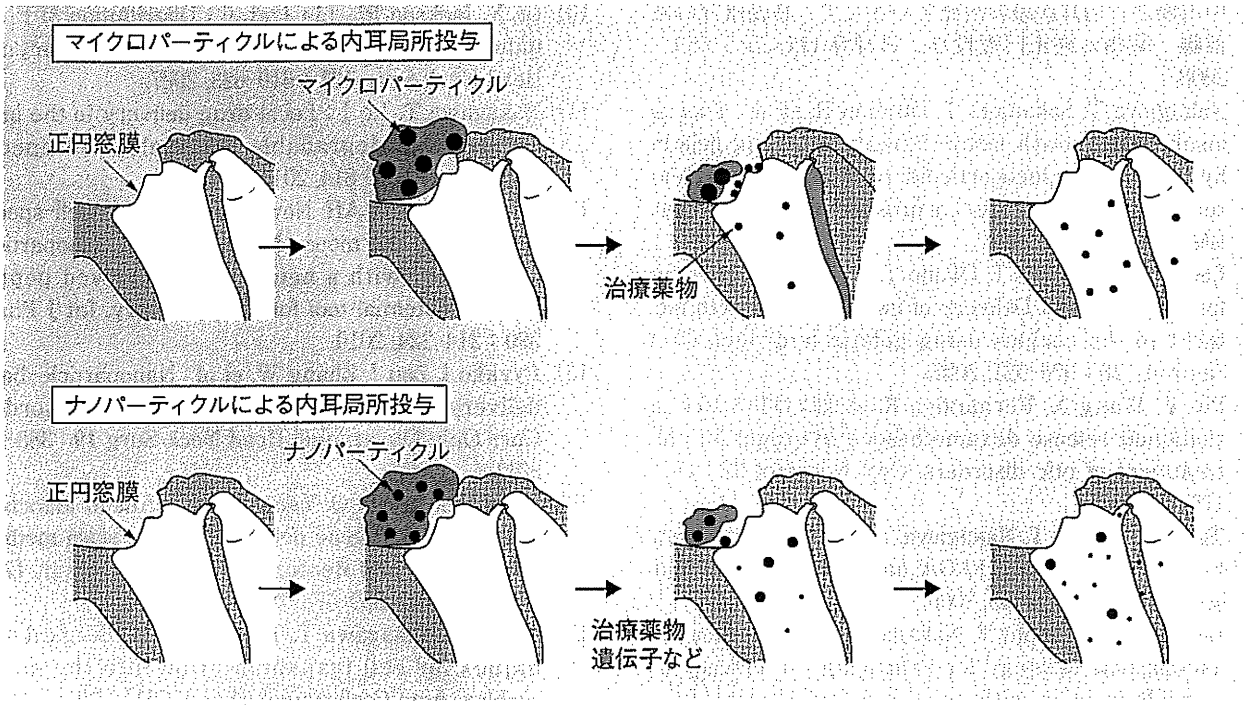

図 1　マイクロパーティクルとナノパーティクルの内耳薬物局所投与における違い（文献 11 から改変）

あり，マイクロパーティクルを正円窓膜上に置いた場合，蝸牛の外でパーティクルが分解されて，薬物が正円窓膜を通ることになるが，ナノパーティクルはパーティクルの状態で正円窓膜を通過し，蝸牛の中で薬物が放出されることになる（図1）[11]。

最近の報告では，ナノパーティクル表面を修飾することにより，投与したナノパーティクルがラセン神経節細胞内に取り込まれることが示されている[12]。経正円窓膜投与では，外リンパ領域には，ある程度安定してナノパーティクルを送達することが可能となりつつあるが，中央階への送達は困難とされている[13]。しかしながら，蝸牛内リンパ電位を測定する技術があれば，齧歯類の小さな蝸牛であっても聴力を損なうことなく，ウイルスベクターを中央階に送達することが可能であることが示されている[14]。

過去にわれわれは，細胞移植を同様の方法で行った場合，聴力低下が起こることを報告しているが[15]，この違いは，投与する物質の大きさの違いと解釈できる。すなわち，マイクロスケールの細胞を中央階に投与すると機能障害は逃れられないが，ナノスケールのウイルスであれば，機能障害は回避できるといえる。これは，将来の内耳再生医療を見据えた場合のナノパーティクルの大きなアドバンテージといえるかもしれない。

現在，さまざまな材料を使ったナノパーティクルの内耳への応用が研究されている[11]。どのような材料を使うかは，ナノパーティクル表面の修飾と合わせて，どのような薬物あるいは遺伝子をどの細胞に送り込むのかによって変わる。急性感音難聴を含めた内耳障害治療に対する内耳再生を含めた新規治療法を臨床へとトランスレーションする段階で，ナノ DDS は重要な役割を担うことが予想される。

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Otol Jpn 21(2): 172-178, 2011 パネルディスカッション1「鼓室内注入療法で感音難聴は治るか？」特集

内耳DDSを用いたIGF1投与による突発性難聴治療

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Local application of IGF1 using inner ear drug delivery system for treatment of sudden deafness

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Recently, local drug application has gained particular attention as a therapeutic strategy for inner ear diseases. In local treatment, therapeutic agents are applied on the round window membrane (RWM) in the middle ear, and transferred into the cochlear fluid through the RWM by diffusion. Sustained delivery of drugs is included critical issues for the efficiency of local therapy. We have performed a clinical trial to test the safety and efficacy of local application of insulin-like growth factor 1 (IGF1) using gelatin hydrogels, which enables sustained release of growth factors, for patients with sudden deafness resistant to systemic steroids. The results demonstrate hearing improvement in a half of patients and no occurrence of serious adverse events, indicating the safety and efficacy of local IGF1 treatment via gelatin hydrogels for sudden deafness.

Key words：drug delivery, growth factor, clinical trial, sensorineural hearing loss, hair cell
和文キーワード：薬物徐放，細胞増殖因子，臨床試験，感音難聴，有毛細胞

論文要旨

中耳正円窓膜を介した内耳への薬物局所投与においては、薬物の適正な効果発現には、薬物の徐放が必要であることが示唆されており、必要な量の薬物を必要な時間供給するドラッグデリバリーシステム（DDS）の応用が、治療効果に大きく貢献すると考えられている。DDSには、いくつかの種類が開発されているが、細胞増殖因子などのポリペプチドでは、ゼラチンハイドロゲルが優れた徐放能力を示す。われわれは、インスリン様細胞増殖因子1（IGF1）をゼラチンハイドロゲルを用いて、内耳局所投与する方法の急性高度難聴に対する有効性を検証するためのトランスレーショナル研究を行い、最近臨床試験を行った。本治療法は、ステロイド全身投与無効の急性高度難聴例の聴力改善に有効であることが示唆され、重篤な有害事象が認められなかったことから、安全性も高いことが示唆された。今後、ランダム化対照試験を行い、さらに有効性を検証する。

背　景

難聴は、先天性、後天性ともに頻度の高い身体障害の要因であり、社会生活の大きな問題となる。難聴は、伝音難聴、感音難聴および混合性難聴に分類することができるが、感音難聴については、有効な治療法がきわめて限られている。聴力低下が固定した感音難聴に対しては、補聴器あるいは人工内耳の装用が選択できる治療法となり、有効な薬物療法はないのが現状である。有毛細胞を含めた蝸牛の細胞は再生能力に乏しく、一旦障害が固定すると、再生することは困難である。したがって、完全に蝸牛の細胞が死に至る前に、自己修復能力を高めてやり、細胞死から救済することが現実的な戦略といえる。突発性難聴に代表される急性高度難聴では、ステロイド全身投与が第一選択とされており、約80%の症例ではなんらかの聴力改善が認められる[1]。しかし、自然治癒傾向を示す症例も少なくないことから[2]、ステロイド全身投与の効果がどこまで聴力改善に貢献しているかは、不明である。最近は、ステロイド大量投与での重篤な副作

用のリスクを考慮し、経口ステロイドを第一選択とすることも提案されている[3]。一方、突発性難聴症例の約2割では、ステロイド全身投与に全く聴力改善が認められないことから[1]、新たな治療方法の研究開発が強く望まれている。

ドラッグデリバリーシステムの必要性

ドラッグデリバリーシステム（DDS）は、体内の薬物分布を量的、時間的、空間的に制御する技術を示し、具体的には、薬物の徐放やターゲッティングを企図し、薬物自体の加工、あるいは、投与方法をモディファイするテクノロジーである。薬物が投与されても、標的となる臓器や細胞に薬物が到達しなければ、効果は発揮されない。到達しても、必要な量が、必要な時間供給されなければ、期待される効果はえられない。しかし、大量、長期投与を行えば、副作用発現のリスクは高くなる。DDSを用いることにより、標的となる臓器に選択的に必要最小限の薬物を徐放することができれば、治療効果を向上させると同時に、副作用発現のリスクを抑制できる。このような観点から、DDSは、副作用発現が問題となる抗がん剤投与や再生医療における細胞増殖因子投与を中心として、近年急速に発展しつつある。内耳は、血流量が少なく、また、中枢神経と同様に薬物の移行を妨げる血液－内耳関門が存在することから、薬物が到達しにくい臓器ととらえることができ、内耳障害治療もDDS応用のよい適応といえる。

ステロイドの全身投与は、突発性難聴治療の第一選択とされているが、動物実験で全身投与されたステロイドのごくわずかが、きわめて限られた時間しか蝸牛に到達していないことが示されている[4]。全身投与でもDDSを応用することにより、蝸牛に長時間、多くの量のステロイドを供給可能となり、音響外傷に対する治療効果が向上する[4]。一方、内耳への局所投与でもDDSの果たす役割は大きい。動物実験やコンピューターシミュレーションで中耳正円窓に投与された薬物の蝸牛内への移行動態が示されている[5),6]。単純な鼓室内投与では、ごく短時間、基底回転のみに薬物は供給されるにすぎないが、ポンプで継続的に薬物を投与することにより、蝸牛の中回転以上に薬物が到達することが示されている。したがって、薬物の局所投与においても、なんらかのDDSを用いることが、その効果を高めるために必要と考えられる。

局所投与の利点と問題点

蝸牛に到達する血流量は限られており、全身投与した薬物のごく一部しか蝸牛に到達しないことから、局所投与、すなわち、鼓室内投与の有用性は、古くから注目されていた。しかしながら、標準的な治療法としての地位を確立するには至らなかった。その要因として、単純に鼓室内投与しただけでは、安定して蝸牛内に薬物を移行させることができないことがあげられる。先述した薬物徐放の必要性の他に、中耳、特に正円窓窩の解剖学的な特徴に関連する事項を考慮しなければならない。第一に、中耳腔内での滞留性が問題となる。中耳腔内の薬物は、粘性が低ければ、容易に耳管から排泄されてしまう。すなわち、ある程度以上の粘性を持たせて、正円窓窩にとどまる工夫が必要となる。また、確実な正円窓窩への投与も問題となる点である。正円窓窩の骨は、正円窓膜を覆い隠すようにオーバーハングしており、さらに、正円窓膜は鼓膜に平行ではなく、直行する向きにある[7]。すなわち、正円窓膜を経鼓膜的に直視することは困難であり、正円窓窩に膜様構造物が存在することがあり、直接正円窓膜を観察できない場合もある[7]。したがって、経鼓膜的に正円窓窩に薬物を確実に留置するためには、内視鏡での確認などなんらかの工夫が必要である。古典的ともいえる鼓室内投与を現代の医療として発展させるためには、少なくとも1）鼓室内での滞留性、2）正確な正円窓窩への投与、そして、先の項でも述べた薬物の徐放という3点に留意し、安定した蝸牛内への薬物移行を実現しなければならない。

経正円窓投与における薬物徐放

経正円窓薬物投与における薬物徐放として、どのような方法が応用可能なのか、多くの研究がなされている。臨床応用がすでに行われている方法として、埋め込み型ポンプと本稿で紹介するゼラチンハイドロゲルがある。中耳あるいは内耳にポンプ先端部を埋め込む方法は、動物実験では広く用いられている方法であるが、埋め込み型ポンプを用いてステロイド投与を行った臨床試験もすでに行われている[8),9]。ポンプに接続したカテーテルの尖端を正円窓窩に留置し、リザーバーを体外に留置し、薬物の送達は浸透圧ポンプにより行われる。バイオマテリアルと比較した場合、埋め込み型ポンプの最大の利点は、薬物の送達速度が一定にできる点にある。バイオマテリアルを用いた薬物徐放では、一般に最初に多量の薬物が放出され、やや速度の落ちた第2段階があり、なだらかな徐放曲線を描く第3段階があるというように、経時的に放出される薬物の量が変化する。一方、必要な手術侵襲は、バイオマテリアルの正円窓窩留置に比較する

と、ポンプの留置の場合、鼓室形成術なみの侵襲となり、さらに、留置されたカテーテルを除去するための手術侵襲も考慮しなければならない。生体分解性のバイオマテリアルを用いれば、体外に除去することを考える必要はなくなる。したがって、投与薬物の性質上、薬物の送達安定度が治療効果に不可欠であり、しかも、その治療効果が大きい場合には、埋め込み型ポンプを用いるメリットが生じるが、臨床的な有効性が大きく変わらないのであれば、バイオマテリアルを用いる方法が汎用される可能性が高い。

バイオマテリアルを用いた内耳薬物投与に関する多くの研究報告がなされている。投与薬物としては、ステロイドが用いられることが多く、ステロイド徐放に適した脂溶性ポリマーに薬物を封入する方法が最も多く用いられている[10)~15)]。このような脂溶性ポリマーは、ステロイドやリドカインの徐放に適したシステムといえるが、水溶性で分子量の大きい神経栄養因子や細胞増殖因子のようなタンパクやペプチドに用いることは、困難である。一方、ゼラチンポリマーからなるゼラチンハイドロゲルは、ゼラチンポリマーの網目に静電気力で薬物が結合するシステムであることから、水溶性で陰性か陽性に荷電しており、比較的分子量の大きな薬物の徐放に適する[16)]。さらに、薬物とゼラチンポリマーの結合に特別な製造工程を必要としないため、製造工程で生物学的な性質が変化する可能性も低い。ゼラチンポリマーの他にも、生態分解性の材料として、ヒアルロン酸、キトサンによる徐放の内耳への応用も報告されているが[17),18)]、神経栄養因子や細胞増殖因子の徐放には応用されていない。ゼラチンハイドロゲルは、これまでに脳由来神経栄養因子(BDNF)、肝細胞増殖因子、インスリン様細胞増殖因子1 (IGF1) の経正円窓膜投与による内耳障害治療において、その有効性が示されている[19)~23)]。特に、BDNFとIGF1については、経正円窓膜投与後の蝸牛外リンパへの徐放が確認されており[19),21)]、現在のところ、バイオマテリアルによる内耳への神経栄養因子や細胞増殖因子の徐放において、唯一有効性が確認されているバイオマテリアルといえる。

ゼラチンハイドロゲルを用いたIGF1局所投与：前臨床試験

神経栄養因子や一部の細胞増殖因子が、強い内耳保護作用を有することは、古くから注目されており、BDNFの強力なラセン神経節細胞の生存促進効果は、人工内耳治療の有効性を高めるとの観点から、積極的に研究が行われている[24)~26)]。神経栄養因子や細胞増殖因子が生物学的効果を発揮するためには、少なくとも数時間、局所に因子が持続的に供給されなければならず、何らかの徐放システムが必要であり、過去の研究では、埋め込み型ポンプやウイルスベクターを用いた遺伝子導入による長期投与が用いられていた。われわれは、まず、ゼラチンハイドロゲルの内耳薬物投与システムとしての有効性を調べるために、BDNFを投与薬物とし、埋め込み型ポンプを用いてBDNFを投与した場合のラセン神経節細胞保護効果[25)]との比較検討を行った[19)]。結果、BDNFをゼラチンハイドロゲルで投与した場合でも、埋め込み型ポンプを使用した場合と同等の組織学的、機能的ラセン神経節細胞保護効果が認められた[19)]。この結果から、内耳へ神経栄養因子や細胞増殖因子を内耳投与する方法として、ゼラチンハイドロゲルが埋め込み型ポンプと同等の有効性を持つことが示唆された。次の段階として、急性障害モデルで有毛細胞保護効果が期待でき、なおかつ、直ちに臨床で使用できるIGF1に着目した研究を行った。

IGF1は、蝸牛の発生に深く関与していることが知られており[29),30)]、過去の基礎的研究では、蝸牛の保護作用が期待できる細胞増殖因子であることが示唆されていた[31),32)]。さらに、近年では、老人性難聴の進行防止に有用であることを示唆する報告もなされている[33)]。しかしながら、in vivoでのIGF1投与による効果の検討は、なされていなかった。まず、IGF1の蝸牛有毛細胞保護効果を検証する目的で、より高い効果が期待できる音響外傷前投与による実験を行った[20)]。IGF1が蝸牛内で作用する可能性がある細胞は、いくつか考えられるが、音響外傷に対して最も脆弱とされている外有毛細胞を組織学的評価の対象とした。結果、ゼラチンハイドロゲルを用いたIGF1局所投与は、高い外有毛細胞保護効果を示し、聴覚閾値の上昇をほぼ完全に抑制することが分かった[20)]。次に、ゼラチンハイドロゲルによる蝸牛外リンパへのIGF1徐放を確認し、音響外傷後に投与した場合の治療的効果および容量依存性を調べた[21)]。音響外傷後に投与した場合、音響外傷前投与に比べると、効果は減弱したが、統計学的に有意の保護効果が確認され、この効果は容量依存性であった。また、同時に有害事象として、局所での炎症所見などを検討したが、明らかな有害事象は認められなかった。さらに、より突発性難聴の病態を反映するモデルと考えられる内耳虚血再還流障害モデルでも、ゼラチンハイドロゲルを用いたIGF1局所投与の有効性検証を行い、有毛細胞保護効果、聴覚保護効果が確認された[22)]。同時に、ヒト側頭骨標本を用いて、経鼓

膜的に、確実に正円窓窩に薬物を留置することを目的として、涙道観察用に開発された超細径内視鏡の応用検討を行い、約2 mmの鼓膜切開を後下象限におくことにより、正円窓窩の観察が可能であることを確認した[34]。

ゼラチンハイドロゲルを用いたIGF1局所投与：プロトコル作成

臨床試験のプロトコル作成にあたっては、科学的意義があり、次のステップの臨床試験に進むために必要十分なエビデンスが提供できるデザインが求められる。さらに、実施可能なデザインであること、実際の臨床的見地から倫理的な配慮がなされていることが重要となる。倫理的な配慮としては、有効性が明確な既存の治療法を受ける機会を損なわないことに留意し、対象をステロイド全身投与が無効な突発性難聴症例とした。診断、効果判定については、厚労省班研究の基準を用いることとし、ステロイド全身投与を7日以上行い、厚労省基準で不変と判定された症例を対象とすることとした。また、発症から長期経過した突発性難聴症例では、薬物治療に対する反応がえられにくいことを考慮し、発症30日未満であることを適格基準に加えた。過去の治療成績（ヒストリカルコントロール）を用いた単群試験とした。ヒストリカルコントロールとしては、過去の京都大学でのステロイド全身投与無効例に対する高気圧酸素療法の治療成績を用いた[35]。高気圧酸素療法では、回復以上を有効とした場合の有効割合は、33%であったため、ゼラチンハイドロゲルを用いたIGF1局所投与の期待有効割合を63%と仮定し、片側αエラー0.05、βエラー0.1とすると、二項分布に基づき算出される必要適格症例数は22例となることから、10%の不適格症例を見込み、目標症例数を25例とした[36]。

主要エンドポイントは、純音聴力検査における5周波数（250、500、1000、2000、4000Hz）の平均聴覚閾値の症例登録前（ステロイド全身投与後）から試験治療12週目の変化、すなわち、回復以上か不変とし、副次エンドポイントを24週目までの平均聴覚閾値変化および有害事象の発現割合とした。京都大学大学院医学研究科医の倫理委員会の承認を受け、UMIN Clinical Trials Registryに登録し、症例登録を開始した。IGF1は、アステラス製薬のソマゾン®注射用10mgを使用し、ゼラチンハイドロゲルは、京都大学医学部附属病院薬剤部で院内製剤として作製し、厳密な管理の下に使用した。試験治療は、すべて京都大学医学部附属病院Day Surgery Unitで局所麻酔科に行われ、手術室でソマゾン®注射用10mgを1 mlの生食に溶解し、ゼラチンハイドロゲルに1時間含浸させた後に、正円窓窩にIGF1含浸ゼラチンハイドロゲルを留置した。正円窓窩への留置は、超細径内視鏡を用いて確認した。純音聴力検査は、投与3日目、1、2、4、12、24週目に行った。試験治療から4日間は入院治療とした[36]。

ゼラチンハイドロゲルを用いたIGF1局所投与：臨床試験結果

2007年12月から2009年7月の間に26症例が登録され、1例のみが不適格となり、25症例が試験治療を受けた。25症例すべてで、24週間の観察が施行され、適正にデータを採取することができた。25症例の内訳は、女性13例、男性12例であり、登録時の平均年齢は49歳であった。試験治療は、発症から15－32日に行われており、平均23日であった。登録時の平均聴力閾値は、81.2dBであった[36]。

試験治療後12週目での平均聴力閾値の変化は、48%、12症例で回復以上（著明回復1例）の改善が認められた。P=0.086となり、帰無仮説は棄却されなかったが、かなり高い有効割合が認められた。試験治療24週目では、56%、14症例で回復以上（著明回復1例）の効果が認められ、P=0.015となり、帰無仮説は棄却された[36]。すなわち、ゼラチンハイドロゲルを用いたIGF1局所投与は、ステロイド全身投与が無効な突発性難聴症例に対して、高気圧酸素と同等かより良好な治療効果を有することが示唆された。

有害事象については、すべての症例でなんらかの有害事象が記録されたが、重篤な有害事象は認められなかった。20%以上の症例で認められた有害事象としては、めまい（44%）、吐き気（24%）、外耳道炎（32%）、中耳炎（28%）、感冒（20%）があった。すべての有害事象は、観察期間中に消失した。鼓膜穿孔が残存する症例は認められなかった。また、感音難聴が悪化する症例も認められなかった[36]。以上の結果から、本治療法は前臨床試験で想定されたように安全性が高い治療法であることが示された。

今回の結果は、ゼラチンハイドロゲルを用いたIGF1局所投与が突発性難聴に有効な治療法であることを証明するものではないが、ある一定のエビデンスを構築したものといえる。すなわち、さらに大規模な臨床試験で有効性を検証する価値がある治療法であることを示すものといえる。

今後の展望

今後の展望として、3つの課題がある。ひとつは、今回の臨床試験の結果に立脚し、有効性を検証する臨床試験を行うことである。有効性の検証としては、一般的にいえば、プラセボを用いたランダム化二重盲検試験が望ましいが、突発性難聴という疾患の特徴、すなわち、治療開始までの時間が予後に関連するという点、また、ゼラチンハイドロゲルを用いたIGF1局所投与が外科的手技を伴う治療であることを考慮すると、プラセボの使用、二重盲検試験は困難といえる。このような観点から、ステロイド無効突発性難聴に対しての治療法として、広く用いられ、エビデンスが集積しつつある治療法であるステロイド鼓室内投与を対照治療としたランダム化臨床試験をデザインした。プロトコルは倫理委員会に承認され、現在、多施設共同試験として施行準備中であり、近く登録受け付けを開始する予定である。

基礎的研究者としての立場から見ると、今回の臨床試験の結果は、動物実験の結果と完全に一致するものではなく、臨床試験でえられた結果を基礎的研究にフィードバックして、研究を進めるべき点がいくつか考えられる。ひとつは、分子生物学的なIGF1の作用機序であり、より詳細な作用機序を明確にすることは、新薬開発につながる可能性がある。また、齧歯類では、傷害後に投与する場合のいわゆるtherapeutic time windowが一般的に数日であるのに対して、臨床試験では3週間以上経過してからの投与で、効果が認められたという点も興味深い点である。傷害された有毛細胞の自己修復の促進以外の治療効果メカニズムも考慮する必要がある。

もうひとつは、感音難聴病態診断法の開発の必要性である。突発性難聴という病態が明確でない疾患を対象とする限り、治療すべき明確な標的が不明瞭となる。これでは、より詳細な作用機序が動物実験で明らかにされても、臨床に反映することが困難となる。治療法の開発と並行して、臨床的な、より詳細な蝸牛病態解析の方法の開発を行うことも、重要な課題といえる。

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Liposome-Encapsulated Hemoglobin Alleviates Hearing Loss After Transient Cochlear Ischemia and Reperfusion in the Gerbil

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Abstract: To test liposome-encapsulated hemoglobin (LEH) in transient cochlear ischemia/reperfusion as a model of sudden deafness, Mongolian gerbils were randomly assigned to receive 2 mL/kg of either low-affinity LEH (l-LEH, $P_{50}O_2 = 40$ mm Hg), high-affinity LEH (h-LEH, $P_{50}O_2 = 10$ mm Hg), homologous red blood cells (RBCs), or saline (each group $n = 6$) 30 min before 15-min occlusion of the bilateral vertebral arteries and reperfusion. Sequential changes in hearing were assessed by auditory brain response 1, 4, and 7 days after ischemia/reperfusion, when the animals were sacrificed for pathological studies. h-LEH was significantly more protective than l-LEH in suppressing hearing loss, in contrast to RBC or saline treatment, at 8, 16, and 32 kHz, where hearing loss was most severe ($P < 0.05$ between any two groups) on the first day after cochlear ischemia/reperfusion. Thereafter, hearing loss improved gradually in all groups, with a significant difference among groups up to 7 days, when morphological studies revealed that the inner hair cells but not the outer hair cells, were significantly lost in the groups in the same order. The results suggest that pretreatment with h-LEH is significantly more protective than l-LEH in mitigating hearing loss and underlying pathological damage, in contrast to transfusion or saline infusion 7 days after transient cochlear ischemia/reperfusion. **Key Words:** Artificial oxygen carrier—Auditory brain response—Cochlear ischemia—Sudden deafness—Reperfusion injury.

Acute interruption of blood supply is considered to be the primary cause of sudden deafness (1), a rare but serious otological event that affects 20 people per 100 000 per year (2). Effective treatments for this condition have, however, been quite limited (3,4), and approaches for improving the demand/supply balance of oxygen (O_2) by local hypothermia (5,6) to reduce demand or hyperbaric O_2 therapy to increase supply remain controversial. Thus, we tested the effects of liposome-encapsulated hemoglobin (LEH) (7,8), the efficacy of which has been reported in reducing focal ischemic injury of rat brain (9–11) as well as of nonhuman primates (12) as an artificial O_2 carrier, but not as a substitute of red blood cells (RBCs) for transfusion (13,14). The liposome capsule is small enough (230 nm) to circulate with plasma through capillaries and collaterals, thereby shortening O_2 diffusion distance in ischemic tissues. At the same time, LEH is large enough to remain in the vascular lumen, extending its retention time and avoiding direct contact of hemoglobin with the vascular endothelium (7,8). As the Mongolian gerbil lacks the posterior communicating arteries of the circle of Willis, occlusion of the bilateral vertebral artery causes hindbrain ischemia; this model has been used as an animal model of transient cochlear ischemia (5,6,15,16). In this study, we examined the effects of LEH with high- and low-O_2 affinity on ischemia and reperfusion injury to the cochlea in terms of hearing loss as determined by auditory brain

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response (ABR) and the severity of damage to the inner and outer hair cells (OHCs) in the gerbil as a simulation of clinical sudden deafness.

MATERIALS AND METHODS

The experiments were conducted in accordance with the Guidelines for Animal Experimentation at Ehime University Graduate School of Medicine. The animals received humane care as required by the institutional guidelines and the *Guide for the Care and Use of Laboratory Animals* (17).

LEH

The relevant characteristics of LEH (Terumo, Tokyo, Japan) have been reported elsewhere (7). Briefly, the liposome capsule, 230 nm in mean diameter, contains purified hemoglobin from outdated human RBCs. The liposome capsule is coated with polyethylene glycol to reduce mutual aggregation, to avoid recognition by the reticuloendothelial cell system, and to prolong its half-life in the circulation (8). In its preparation, inositol hexaphosphate was used to control O_2 affinity to $P_{50}O_2 = 40$ mm Hg for low-affinity LEH (l-LEH) and to $P_{50}O_2 = 10$ mm Hg for high-affinity LEH (h-LEH) (Fig. 1). LEH, suspended in saline at a hemoglobin concentration of 6 g/dL or 20% by volume, has reduced viscosity (2 cP) compared to blood (5 cP), and specific gravity close to that of plasma. Homologous RBCs were washed with saline three times and suspended in saline at a final concentration of 20% hematocrit.

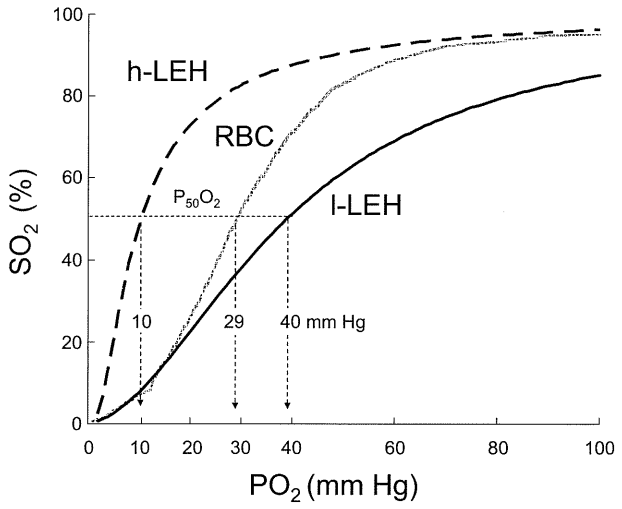

FIG. 1. Oxygen dissociation characteristics for h-LEH, l-LEH, and gerbil's RBC. Oxygen dissociation characteristics have been determined for every batch of LEH by actually measuring O_2 saturation using a UV-Spectrophotometer (MPS-2000, Shimadzu, Kyoto, Japan) in closed cells under a varying range of O_2 (0 to 95%) and 5% CO_2 with N_2 replacement. Iron oxidation state was determined by cyanmethemoglobin method. The equilibrium curve for RBC was from gerbil's RBC ($P_{50}O_2 = 28.7$ mm Hg). While l-LEH ($P_{50}O_2 = 40$ mm Hg) is considered to have higher O_2 delivery than gerbil's RBC ($P_{50}O_2 = 28.7$ mm Hg) under physiologic conditions, h-LEH ($P_{50}O_2 = 10$ mm Hg) is considered to have more efficient O_2 delivery than gerbil's RBC under hypoxic condition.

Experimental animals

Adult male Mongolian gerbils (*Meriones unguiculatus*) weighing 60–80 g were purchased from Kyudo (Tosu, Japan) and used at 12–16 weeks of age. Anesthesia was induced with a mixture of 3% halothane and nitrous oxide:oxygen (7:3) gas, and was maintained with a mixture of 1% halothane gas. The animals were ventilated artificially via a transoral tracheal tube (tidal volume 1 mL; respiration rate 70/min). During the experiment, body temperature was monitored with a thermocouple probe (PTI-200, Unique Medical, Tokyo, Japan) placed in the rectum and kept at 37 ± 1°C using a heating pad (HP-1 M, Physitemp, Clifton, NJ, USA). The femoral vein was exposed to establish an intravenous infusion line by polyethylene catheter. The animals were randomly assigned to receive 2 mL/kg of h-LEH ($n = 6$), l-LEH ($n = 6$), RBCs ($n = 6$), or saline ($n = 6$) 30 min before cochlear ischemia over 10 min to avoid acute volume load; after which, the catheter was removed and the vein was ligated.

Transient cochlear ischemia and reperfusion

Thirty minutes after administration of the solutions, cochlear ischemia and reperfusion were effected as previously described (15). Briefly, with the animal in supine position, the vertebral arteries were exposed bilaterally (Fig. 2A) and dissected free from the surrounding tissues through a ventral midline incision in the neck. Silk ligatures (4-0) were looped loosely around each artery. Ischemia was then induced in both cochleae by pulling on the ligatures (Fig. 2B) simultaneously using 5-g weights for 15 min. Subsequently, the threads were removed to allow reperfusion, which was confirmed by observation through an operating microscope. The wound was closed and the animals were returned to cages with water and food ad libitum until further testing. As a preliminary study, changes in cochlear blood flow during the bilateral vertebral artery occlusion were monitored by laser Doppler flow meter (ALF-21, Advance, Tokyo, Japan) focused on the lateral aspect of its basal turn in six animals. The percent level of cochlear blood flow was calculated, with the value measured after exsanguination taken as 0% and the preischemic value regarded as 100%.

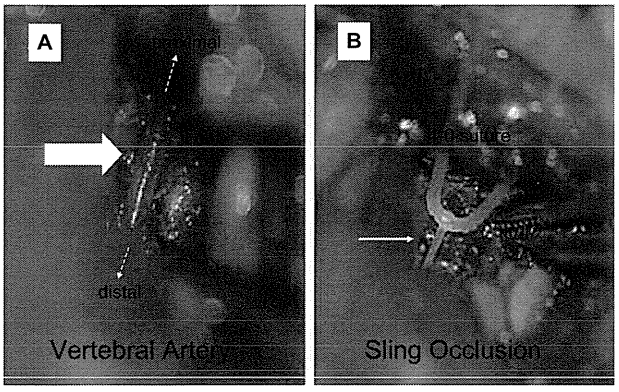

FIG. 2. Intact vertebral artery (A) and occlusion by pulling the sling (B). Silk ligatures (4-0) were looped loosely around bilateral vertebral artery. Ischemia was induced in both cochleae by pulling the ligatures simultaneously using 5-g weights for 15 min. Releasing and removing these slings allowed reperfusion of the cochlear artery.

Evaluation of hearing by ABR

The hearing of each animal was assessed before and at 1, 4, and 7 days after ischemia and/or reperfusion. The animal was anesthetized with an intramuscular injection of ketamine (50 mg/kg) and xylazine (1 mg/kg), and then ABR was recorded using a signal processor (NEC Synax 1200, NEC Medical Systems, Tokyo, Japan). Recording and reference needle electrodes were placed at the vertex and retroauricular area, respectively. The stimulus sound was a pure tone burst at 8, 16, and 32 kHz (rise and fall time, 1 ms; duration, 5 ms; repetition rate, 15/s) delivered in an open field system (DPS-725, DIA Medical, Tokyo, Japan). The phases of the stimuli were alternated, thereby negating interference of the cochlear microphonics. The speaker was located a constant 20 cm from the left external auditory canal. Responses to 1000 consecutive stimuli were averaged. The ABR threshold was determined by recording responses in 5-dB steps.

Histological study

For the histological study, the animals were decapitated under deep anesthesia 7 days after ischemia. After removing the otic bullae, the cochleae were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4 into the scala tympani and postfixed for 2 h with the same fixative at 4°C. The specimens were immersed in phosphate-buffered saline (PBS) and the organ of Corti was dissected using a surface preparation technique under an operating microscope. The walls of the bony cochleae were removed entirely without disrupting the organ of Corti. Then, the basal turn of the organ of Corti was isolated. The specimen was stained with rhodamine-phalloidin (Molecular Probes, Eugene, OR, USA) diluted 250 times in PBS containing 0.25% Triton X-100 and 1% bovine serum albumin for 30 min at room temperature. After rinsing in PBS, it was further stained with Hoechst 33342 (Calbiochem-Novabiochem, La Jolla, CA, USA) dissolved in PBS in a dark room for 1 h. It was again rinsed in PBS and mounted in carbonate-buffered glycerol (one part 0.5 M carbonate buffer at pH 9.5 to nine 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 (Olympus, Tokyo, Japan) equipped with green (band pass filter [BP] 546, Farb Teiler Spiegel [FT] 580, long pass filter [LP] 590 nm) and UV (BP 365, FT 395, LP 397 nm) filters. Rhodamine-phalloidin staining permits observation of the hair cell stereocilia, whereas Hoechst 33342 staining reveals that of the nuclei. The numbers of intact and dead hair cells at the basal turn were counted, and the percentage of dead hair cells to intact hair cells (IHCs) was determined. As gerbils have about 300 IHCs at the basal turn, we examined at least 200 IHCs in each specimen.

Statistics

All data are presented as mean ± SD. Statistical differences between groups were evaluated using the Mann–Whitney *U*-test. The results were considered significant at $P < 0.05$.

RESULTS

Cochlear ischemia

Occlusion of the bilateral vertebral arteries (Fig. 2) severely reduced cochlear blood flow as measured by laser Doppler flow meter (Fig. 3), which then increased over the preischemic level and returned to baseline shortly after reperfusion.

Hearing loss

The preischemic ABR threshold was set at 0 dB, and the subsequent increase (hearing impairment) in threshold is shown on the ordinate (Fig. 4). At 8 kHz (left panel), the increase in ABR threshold was significantly suppressed in the order of the animals treated with h-LEH, l-LEH, and RBCs or saline. Among the frequencies tested (8, 16, and 32 kHz), the higher the frequency, the more severe the magnitude of hearing loss and difference among the treatment groups. The magnitude of hearing loss was most prominent on day 1, then declining in severity with time to day 7 at each frequency tested and in every