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

MECHANISM OF NSAID OTOTOXICITY

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

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

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

POSSIBLE APPLICATION OF NSAIDs FOR COCHLEAR PROTECTION

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

CONCLUSION

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

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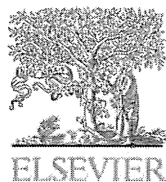

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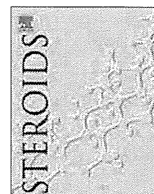

Effects of neuroactive steroids on cochlear hair cell death induced by gentamicin

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ABSTRACT

As neuroactive steroids, sex steroid hormones have non-reproductive effects. We previously reported that 17β-estradiol (βE2) had protective effects against gentamicin (GM) ototoxicity in the cochlea. In the present study, we examined whether the protective action of βE2 on GM ototoxicity is mediated by the estrogen receptor (ER) and whether other estrogens (17α-estradiol (αE2), estrone (E1), and estriol (E3)) and other neuroactive steroids, dehydroepiandrosterone (DHEA) and progesterone (P), have similar protective effects. The basal turn of the organ of Corti was dissected from Sprague–Dawley rats and cultured in a medium containing 100 μM GM for 48 h. The effects of βE2 and ICI 182,780, a selective ER antagonist, were examined. In addition, the effects of other estrogens, DHEA and P were tested using this culture system. Loss of outer hair cells induced by GM exposure was compared among groups. βE2 exhibited a protective effect against GM ototoxicity, but its protective effect was antagonized by ICI 182,780. αE2, E1, and E3 also protected hair cells against gentamicin ototoxicity. DHEA showed a protective effect; however, the addition of ICI 182,780 did not affect hair cell loss. P did not have any effect on GM-induced outer hair cell death. The present findings suggest that estrogens and DHEA are protective agents against GM ototoxicity. The results of the ER antagonist study also suggest that the protective action of βE2 is mediated via ER but that of DHEA is not related to its conversion to estrogen and binding to ER. Further studies on neuroactive steroids may lead to new insights regarding cochlear protection.

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1. Introduction

Recent studies have revealed non-reproductive functions of sex steroid hormones. In the central nervous system, sex steroid hormones are derived from not only circulation but also local synthesis de novo. Considering their synthesis and actions in the central nervous system, they are termed as neurosteroids or neuroactive steroids. Regarding non-reproductive actions of neuroactive steroids, cell protective effects of estrogen, dehydroepiandrosterone (DHEA), and progesterone (P) have been reported in various organs as well as the central nervous system. It has been demonstrated that several enzymes of the pathway synthesizing these hormones are expressed in the brain [1] and the cochlea [2].

Excessive amounts of gentamicin (GM), an aminoglycoside antibiotic, induce cochlear hair cell death, at least in part, through an apoptotic process [3–5]. Previously, we reported that 17β-estradiol (βE2) had protective effects against GM ototoxicity. Using explants of the rat cochlea, we confirmed that βE2 ameliorated the GM-induced apoptotic death of outer hair cells through the inhibition of the c-Jun N-terminal kinase (JNK) pathway [6]. Estrogens are also suggested to exert protective effects against acoustic injury to the mouse cochlea [7]. The protective effects of estrogen against acoustic injury are reportedly mediated by estrogen receptors (ERs) [7].

DHEA and progesterone exert many actions related to neuroprotective effects on the brain. Previous studies have demonstrated that DHEA protects the cochlea against acoustic [8] and ischemia-induced injuries [9] in the guinea pig. However, the protective mechanism of DHEA against cochlear injury has not been clarified. Namely, protective actions of DHEA may be mediated by their conversion to βE2.

In the present study, we examined whether the protective effect of βE2 against GM ototoxicity was mediated by ERs. The protective effects of other estrogens, DHEA and P, on GM ototoxicity were also tested.

2. Experimental

2.1. Culture techniques

The basal turn of the organ of Corti was dissected from Sprague–Dawley rats on postnatal days 3 (p3) to 5 (p5) and cultured according to the methods of Van de Water and Ruben [10] and Sobkowicz et al. [11]. All animal procedures were carried out

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according to guidelines of the Laboratory Animal Research Center of the University of Tsukuba.

Because methods of culturing cochlear explants were described in detail in our previous report [6], a brief resume is given here. Cochlear explants were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 25 mM HEPES, and 30 U/ml penicillin and were cultured in an incubator at 37 °C with 5% CO_2 and 95% humidity. These cultures were maintained in the above-described initial medium overnight (8–12 h) and then were exposed to a medium containing 100 μM GM (Sigma, MO, USA) for 48 h.

Before conducting this study, we examined hair cell loss induced by the exposure of cochlear explants to several concentrations of GM. A medium containing 100 μM GM showed an approximately 70% loss of outer hair cells [6]. In the present experiment, therefore, cochlear explants were subjected to 100 μM GM to assess the effects of neurosteroids.

2.2. Treatment with estrogens

Cochlear explants were pretreated overnight with a culture medium containing βE2 (Sigma, MO, USA) to allow the substance to penetrate hair cell membranes. Each of the 13 explants was then exposed to media containing 100 μM GM and each concentration of βE2 (1 nM–1 μM) for 48 h. The tested concentration of βE2 was determined based on our previous report [6]. βE2 was initially dissolved in ethanol and then diluted in a medium to the final concentration immediately before use.

Cochlear explants were pretreated overnight with a culture medium containing 17α-estradiol (αE2), estrone (E1) or estriol (E3). These agents were purchased from Sigma (St. Louis, MO, USA). Each of the eight explants was then exposed to media containing 100 μM GM and 100 nM estrogens for 48 h. The tested concentration of the estrogens was based on our previous report [6]. αE2 and E3 were initially dissolved in ethanol, and E1 was dissolved in dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO, USA), then diluted in a medium to the final concentration, 100 nM, immediately before use. These solvents were included in the culture media at concentrations of less than 0.1%.

2.3. Treatment with an ER selective antagonist and βE2

The effect of ICI 182,780, an ER selective antagonist [12], on GM ototoxicity was examined using the explants of the basal turn. ICI 182,780 (Tocris Bioscience, UK) was diluted in ethanol and added to the culture medium at each concentration (1 nM–10 μM) immediately before use. The explants were pretreated overnight with a culture medium containing ICI 182,780 plus 100 nM βE2 to allow the substance to penetrate hair cell membranes. Each of the eight explants was then exposed for 48 h to media containing 100 μM GM, ICI 182,780, and E2.

2.4. DHEA treatment

Using the same procedure as that followed for the E2 experiment, each of the eight explants was exposed to media containing 100 μM GM and each concentration of DHEA (MP Biomedicals, France) for 48 h. The tested concentrations of DHEA were 0.1, 1, 10, and 100 nM. DHEA, initially dissolved in ethanol to 10 mM, was diluted in a medium to the final concentration immediately before use.

In addition, we examined whether the effects of DHEA were mediated by its conversion to estrogens and their binding to ERs. Specifically, the effect of DHEA plus ICI 182,780 on gentamicin ototoxicity was tested. Each of the eight explants was exposed to media containing 100 μM GM, 10 nM DHEA, and 10 nM–1 μM ICI 182,780 for 48 h.

2.5. P treatment

Each of the eight explants was cultured in media containing 100 μM GM and 0.1 nM–100 μM P (Sigma, MO, USA) for 48 h. P was initially dissolved in DMSO and was diluted in a medium to the final concentration immediately before use.

2.6. Cytochemistry

After the explants were cultured for 48 h in culture media containing 100 μM GM alone or 100 μM GM plus the neurosteroids, the explants were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min and then permeabilized with 5% TritonX-100 in PBS with 10% FBS for 10 min. The specimens were stained for phalloidin with a conjugated Alexa Fluoro probe (1:100, Molecular Probes, Inc., CA, USA) at room temperature for 1 h. The specimens were observed under a fluorescence microscope (Axiophoto, Zeiss, Germany). Loss of hair cells was expressed as a percentage and compared among the groups. Phalloidin, a specific marker for cellular F-actin, labels stereociliary arrays and the cuticular plates of hair cells.

2.7. ER staining

Localization of ERs in the organ of Corti explants was examined. Explants were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min and rinsed in PBS ($n = 4$). Specimens were permeabilized by 0.2% Triton X-100 (Sigma, MO, USA) and incubated for 30 min at room temperature in 1% fetal bovine serum. They were then incubated with ERα or ERβ antibody (H-184 and H-150, Santa Cruz Biotechnology Inc., CA, USA) at 4 °C overnight. After rinsing in PBS, they were incubated with the secondary antibody (1:100, fluorescein (FITC)-conjugated goat anti-rabbit IgG, Sigma, MO, USA) in PBS at 4 °C for 2 h in darkness. After ER staining, hair cells were stained with the Alexa Fluoro phalloidin probe.

2.8. Data analysis

All data are expressed as mean ± S.E.M. Statistical analysis was performed by unpaired *t*-test or one-factor ANOVA followed by Bonferroni post hoc tests, as required (StatView 5.0). *p*-values less than 0.05 were considered significant. All experiments consisted of $n \geqq 8$ explants per experimental group.

3. Results

3.1. Control study

Before conducting GM experiments, the effects on hair cells of βE2, αE2, E1, E3, DHEA, P, 0.1% ethanol, and 0.1% DMSO as solvents were examined. In the control explants maintained in the initial media containing these agents for 48 h without exposure to GM, almost all hair cells including one row of inner and three rows of outer hair cells were present. Namely, there was no statistically significant hair cell loss when explants were cultured for 48 h in media containing each agent without GM (data not shown). Furthermore, we also confirmed that there was no significant difference in hair cell loss between explants exposed to a medium containing 100 μM GM with 0.1% ethanol or 0.1% DMSO for 48 h and those with a medium containing 100 μM GM alone (data not shown).

3.2. Protection of hair cells with estrogens against GM ototoxicity

We examined the effect of estrogens on GM-induced outer hair cell damage. βE2 treatment significantly decreased outer

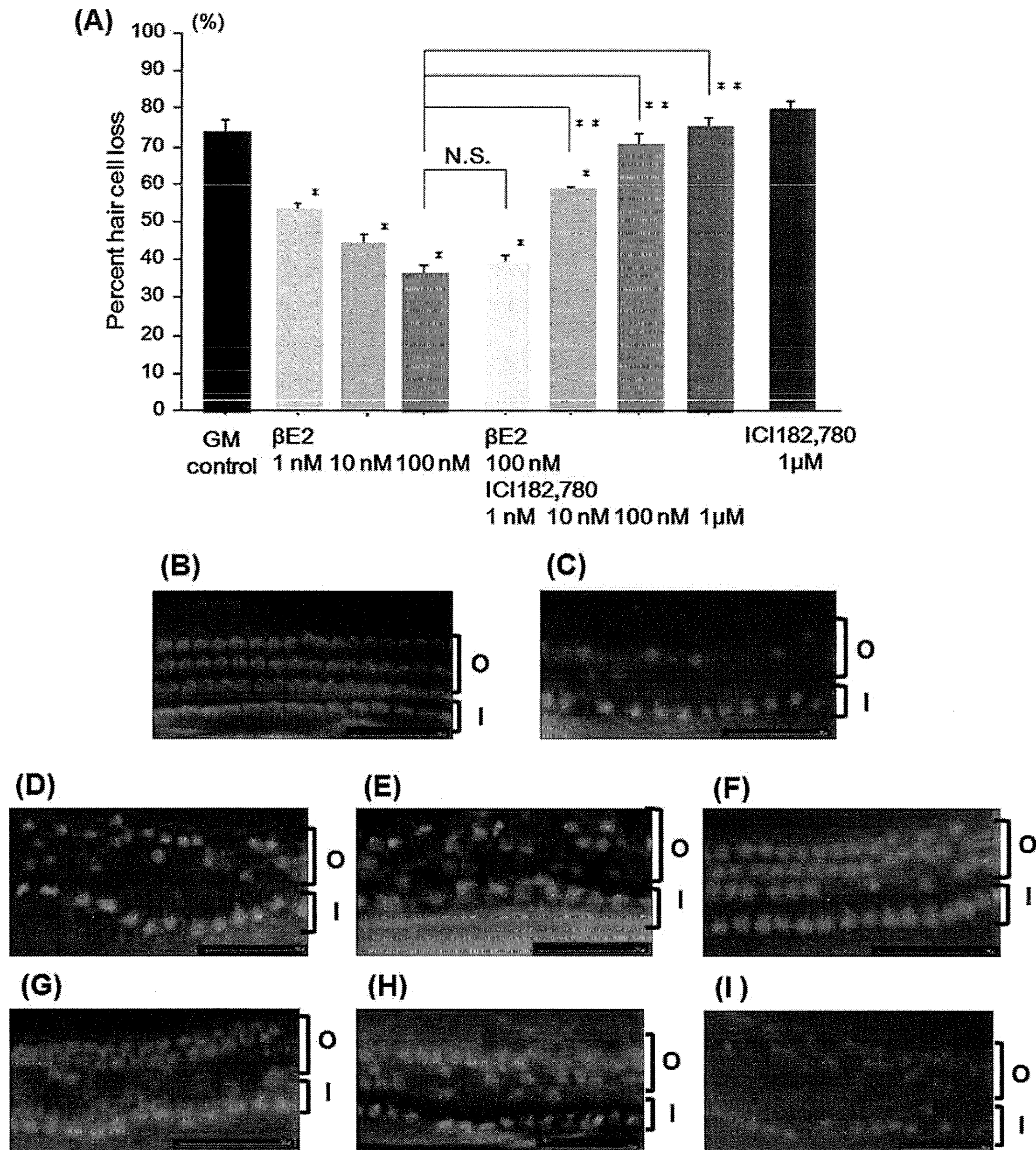


Fig. 1. Effects of βE2 and ICI 182,780 on GM-induced outer hair cell loss. (A) Quantitative analysis of outer hair cell loss. 100 μM GM induced about 70% outer hair cell loss. βE2 dose-dependently decreased GM-induced outer hair cell loss (one-factor ANOVA and Bonferroni post hoc test: $^{*}p < 0.05$ as compared with the GM control group). ICI 182,780 antagonized the protective effect of 100 nM βE2 and affected dose-dependently (one-factor ANOVA and Bonferroni post hoc test: $p < 0.05$). (B–G) Representative photographs of each group. (B) Solvent alone. (C) GM control (100 μM GM alone). (D) 100 μM GM plus 1 nM βE2. (E) 100 μM GM plus 10 nM βE2. (F) 100 μM GM plus 100 nM βE2. (G) 100 μM GM plus 100 μM βE2 and ICI 182,780 1 nM. (H) 100 μM GM plus 100 nM βE2 and 10 nM ICI 182,780. (I) 100 μM GM plus 100 nM βE2 and 100 nM ICI 182,780. O: outer hair cell. I: inner hair cell. Scale bar: 50 μm.

hair cell loss as compared with the control group exposed to GM alone (Fig. 1A, one-factor ANOVA and Bonferroni post hoc test: $^{*}p < 0.05$). αE2, E1, and E3 also ameliorated GM ototoxicity (Fig. 2, one-factor ANOVA and Bonferroni post hoc test: $^{*}p < 0.05$).

3.3. ER expression in the organ of Corti

Immunostaining of culture explants with ERα and ERβ antibodies was examined. In p3–5 rats, both ERα and ERβ were observed in the inner and outer hair cells (Fig. 3). This finding is in agreement with the past report of immature rat [13]. All negative controls did not show any staining of ERs.

3.4. A selective ER antagonist inhibits the protective effect of E2 on GM ototoxicity

Treatment with 1–1000 nM ICI 182,780, a selective ER antagonist, without GM exposure for 48 h did not induce any significant hair cell loss. Furthermore, there was no significant hair cell loss in the culture explants with 100 nM βE2 plus these concentrations of ICI 182,780 without GM exposure. We examined the effects of ICI 182,780 on the protective effects of βE2 against GM-induced hair cell loss. βE2 significantly protected hair cells against 100 μM GM (Fig. 1A and F). Addition of ICI 182,780 antagonized the protective effects of 100 nM βE2. Namely, ICI 182,780 increased hair cell loss as compared with the 100 nM βE2 plus 100 μM GM subgroup (Fig. 1A and F–I, one-factor ANOVA and Bonferroni post

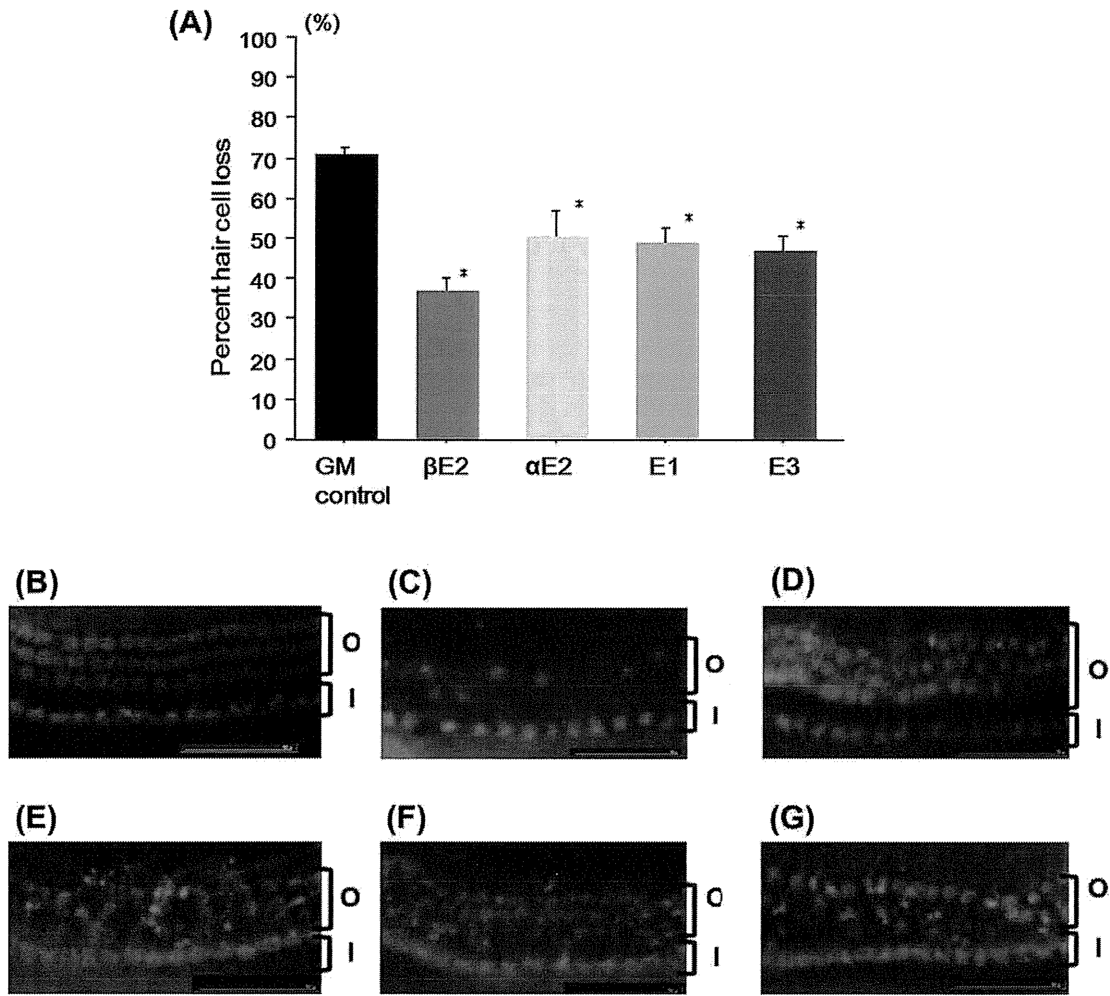


Fig. 2. Effects of estrogens on GM-induced hair cell loss. (A) Quantitative analysis of outer hair cell loss. Each estrogen protected the cochlea (one-factor ANOVA and Fisher post hoc test: $^{*}p < 0.05$ as compared with the GM control group). (B–G) Representative photographs of each group. (B) Solvent alone. (C) GM control (100 μM GM alone). (D) 100 μM GM plus 100 nM βE2. (E) 100 μM GM plus 100 nM αE2. (F) 100 μM GM plus 100 nM E1. (G) 100 μM GM plus 100 μM E3. O: outer hair cell. I: inner hair cell. Scale bar: 50 μm.

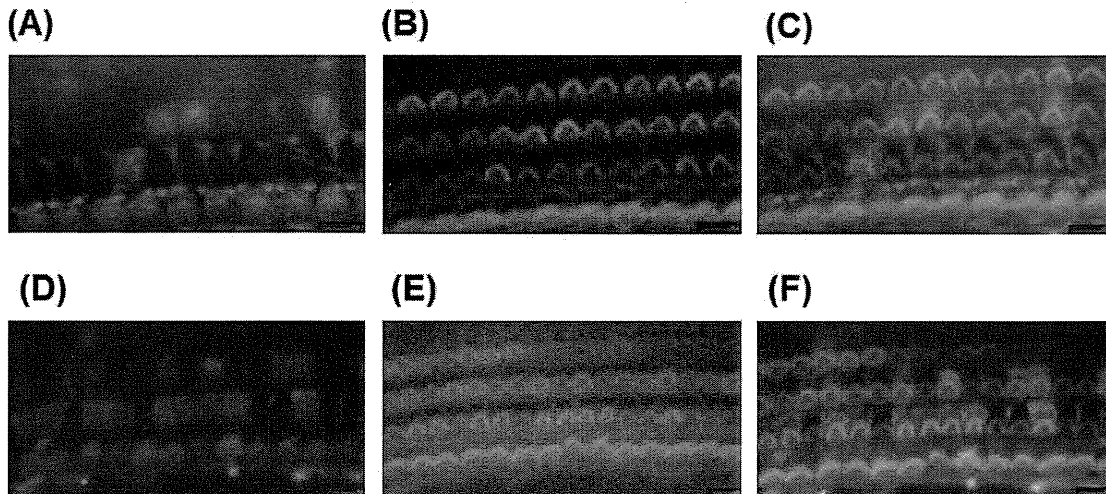
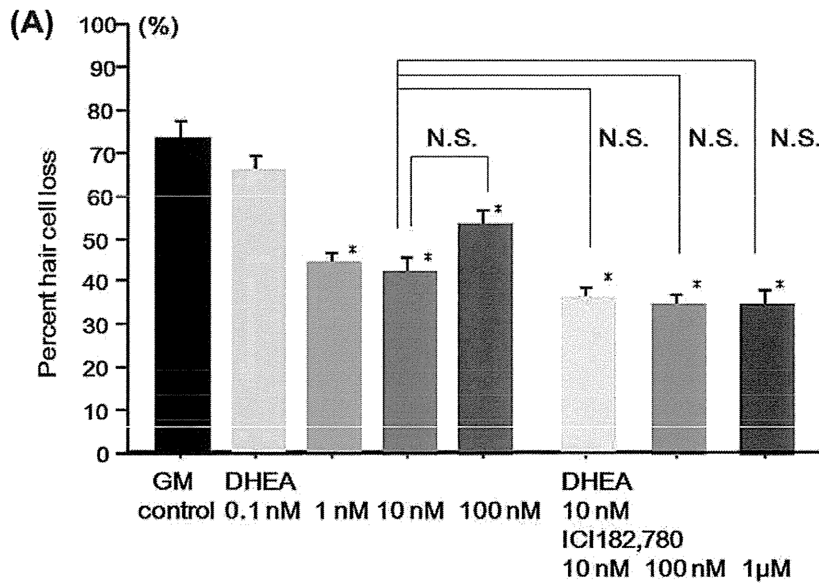


Fig. 3. The localization of ERα and ERβ in the explants. (A) and (D): ERα and ERβ were stained by each antibody (green color). (B) and (E): Hair cells were stained with phalloidin (red color). (C) and (F): Merged images of each ER and hair cells. Outer and inner hair cells were stained with both ERs. Scale bar: 10 μm.

hoc test: $p < 0.05$). The subgroup with 100 nM ICI 182,780, 100 nM βE2, and GM showed almost the same degree of hair cell loss as that of the control group with GM alone. These results suggest that the protective effects of βE2 against GM may be mediated by ERs.



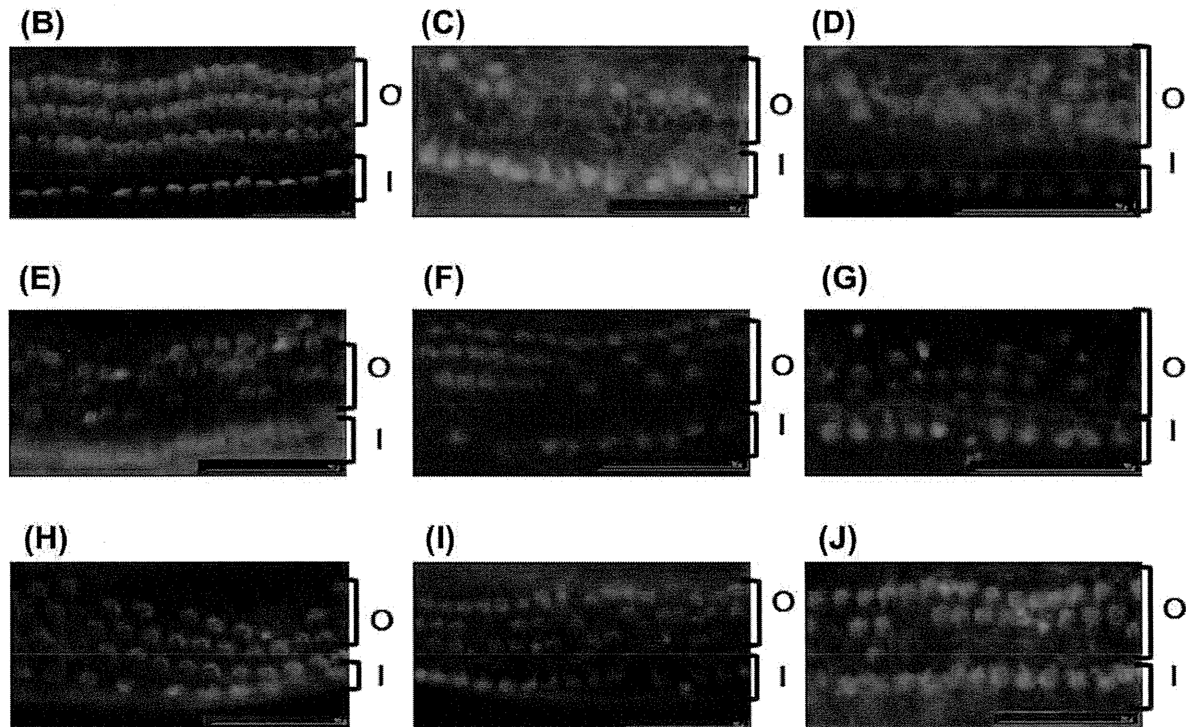


Fig. 4. Effects of DHEA on GM-induced hair cell loss. (A) Quantitative analysis of outer hair cell loss. DHEA protected the cochlea at concentrations of 1–100 nM (one-factor ANOVA and Bonferroni post hoc test: $^*p < 0.05$ as compared with the GM control group). ICI 182,780 did not affect DHEA protection at any concentration. (B–J) Representative photographs of each group. (B) Solvent alone. (C) GM control (100 μM GM alone). (D) 100 μM GM plus 0.1 nM DHEA. (E) 100 μM GM plus 1 nM DHEA. (F) 100 μM GM plus 10 nM DHEA. (G) 100 μM GM plus 100 nM DHEA. (H) 100 μM GM plus 10 nM DHEA and 10 nM ICI 182,780. (I) 100 μM GM plus 10 nM DHEA and 100 nM ICI 182,780. (J) 100 μM GM plus 10 nM DHEA and 1 μM ICI 182,780. O: outer hair cell. I: inner hair cell. Scale bar: 50 μm.

3.5. DHEA protection of outer hair cells against GM ototoxicity is not mediated by ER

The effect of DHEA on GM ototoxicity was examined. DHEA decreased outer hair cell loss at concentrations of 1, 10, and 100 nM compared with the GM control group, although 0.1 nM DHEA did not have any protective effect (Fig. 2).

Treatment with 10 nM–1 μM ICI 182,780 and 10 nM DHEA for 48 h did not induce hair cell loss without GM exposure. In addition, treatment with 10 nM–1 μM ICI 182,780 and DHEA did not affect hair cell loss induced by GM as compared with the DHEA subgroup (Fig. 4A and F–J). These findings suggest that the protective effect of DHEA against GM ototoxicity was not mediated by ER.

3.6. P does not affect GM-induced hair cell loss

The effect of P on GM ototoxicity was examined. In contrast to the protective effects observed in the E2- and DHEA-treated groups, P treatment did not affect hair cell loss induced by GM (Fig. 5).

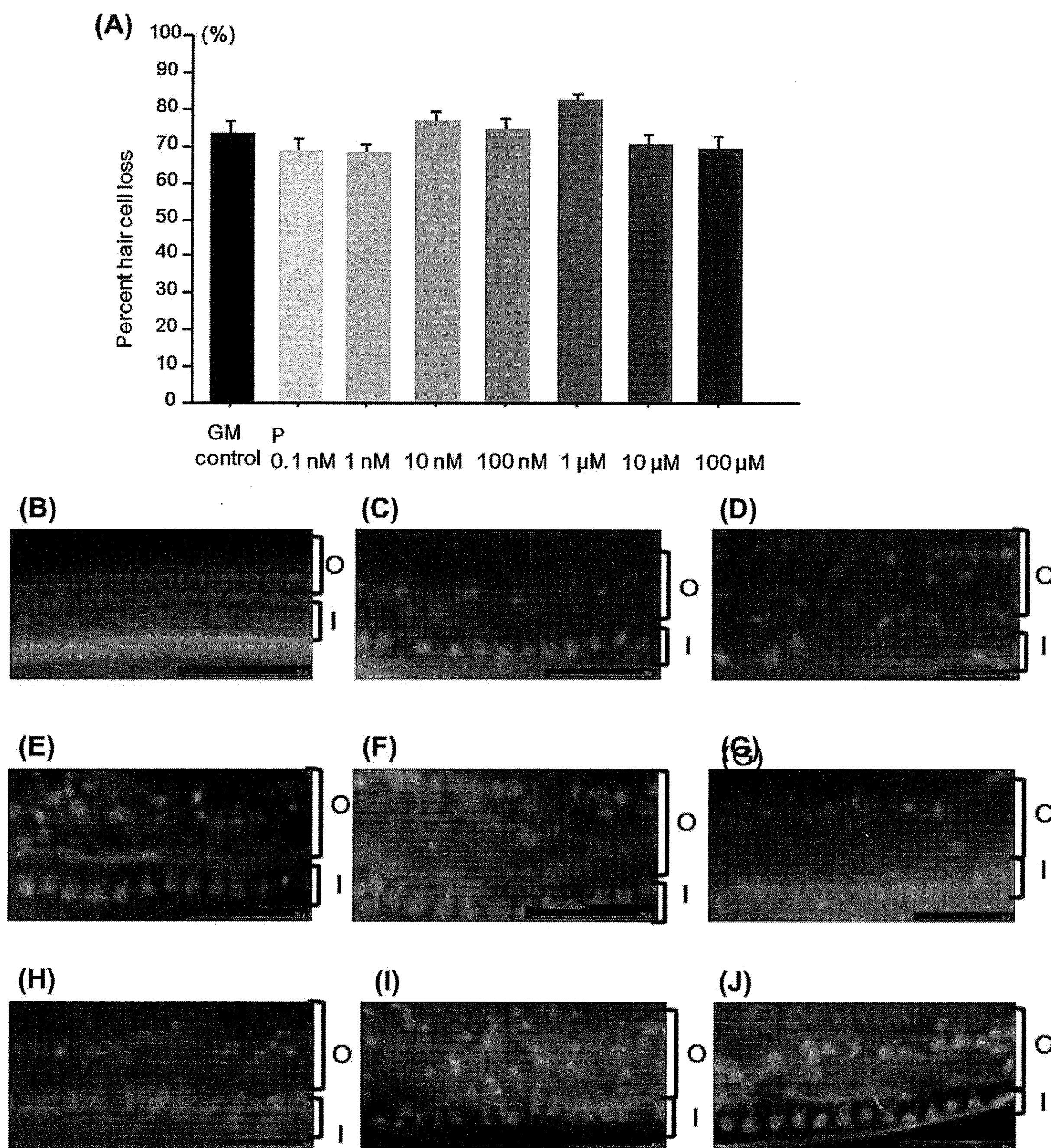


Fig. 5. Effects of P on GM-induced outer hair cell loss. (A) Quantitative analysis of outer hair cell loss. P did not have any effect on GM-induced outer hair cell loss at concentrations of 0.1 nM–100 μM. (B–J) Representative photographs of each group. (B) Solvent alone. (C) GM control (100 μM GM alone). (D) 100 μM GM plus 0.1 nM P. (E) 100 μM GM plus 1 nM P. (F) 100 μM GM plus 10 nM P. (G) 100 μM GM plus 100 nM P. (H)100 μM GM plus 1 μM P. (I) 100 μM GM plus 10 μM P. (J) 100 μM GM plus 100 μM P. O: outer hair cell. I: inner hair cell. Scale bar: 50 μm.

4. Discussion

Estrogens regulate cellular functions through genomic and nongenomic pathways. Estrogens bind to ERs and affect gene expression in the classical genomic pathway. In addition, ERs are also involved in some types of non-classical pathways [14]. To date, two isoforms of ER have been identified: ERα and ERβ. Both subtypes of ER are present in most organs of the body, but the biological differences between the subtypes are unclear. At present, no difference related to gender or age has been shown as for the expression pattern of the receptors [15]. Both receptors are present in the cochlea of rodents and humans, and areas responsible for sound transmission (inner and outer hair cells and spiral ganglion cells) and for inner ear homeostasis (stria vascularis and spiral ligament) were positive for ERs [16,17]. Our immunohistochemical studies revealed that both subtypes of ERs were present in the culture explants.

βE2 protected the cochlea against GM ototoxicity, as we reported in the previous [6] and the present studies. ICI 182,780 is an ER antagonist that blocks both subtypes [18] and reportedly antagonizes both nuclear ER and extranuclear ER, which can exert rapid nongenomic signaling [18–21].

ER beta has been considered to be of particular importance in the cochlear protection of βE2 from acoustic injury because ER beta but not ER alpha knockout mice are vulnerable to acoustic injury [7]. Further studies will be necessary to clarify biological differences between the two ER subtypes.

Natural estrogens have three different forms (E1, E2 and E3). Of these, βE2 is the most potent estrogen. Our findings revealed that all three estrogens have protective effects against gentamicin ototoxicity. αE2, the natural enantiomer of βE2, does not generally exist in humans and is considered to be less active on ERs than βE2 [22]. However, αE2 reportedly exerted protective effects against oxidative stress, ischemic injury, Alzheimer's disease and Parkinson's disease in the central nervous systems [23–26] although the precise action of αE2 has not been clarified. αE2 exhibited protective effects on the cochlear culture explants in the present study. Further experiments may be necessary concerning the protective mechanism of αE2 in the cochlea.

DHEA is one of the most abundant neurosteroids synthesized de novo in the nervous system [27]. Its neuromodulatory actions, including regulation of the activity of gamma-aminobutyric acid (GABA) and N-methyl-D-aspartate (NMDA) receptors [28,29], are known. DHEA is also an agonist of an intracellular receptor, sigma 1, which is involved in memory processes, stress responses, depression, neuroprotection, and pharmacodependence [30]. In addition, DHEA exhibits antioxidant effects in the central nervous system [31].

Previous studies of the auditory system demonstrated that DHEA sulfate protected the cochlea against acoustic [8] and ischemia-induced injuries [9]. In the present study, we revealed that DHEA also had protective effects against GM ototoxicity to the outer hair cells. At present, the mechanism underlying the cochlear protective activity of DHEA is unknown. Through the steroid synthesis and metabolism pathway, DHEA is converted to testosterone and, in turn, to βE2. Because the protective effect of DHEA revealed in the present study was not inhibited by ICI 182,780, it seems unlikely that the cochlear protection of DHEA is mediated by its conversion to βE2 and binding to ER.

P is another neurosteroid which, along with its metabolites, is demonstrated in the human brain [32,33]. Schumacher et al. [34], suggesting that P was a particularly effective agent for the treatment of traumatic injury to the nervous system, provided five characteristics of P: a large therapeutic window, sensitivity in the aging nervous system, protective effects in both males and females, few negative effects, and no harmful effects in the peripheral tissue. A recent study has shown that P synthesis increases in response to injury, which is consistent with the important roles of protecting and regenerating the nervous system [35–37]. On the other hand, there are some controversies regarding the protective effects of P in the central nervous system [38,39]. It has been reported that P decreases the neuroprotective effects of βE2 [40–42].

The present results suggest that P does not have any effect on GM ototoxicity. P protected neurons of the central nervous system against glutamate-induced toxicity at concentrations of 3–100 nM [43,44] and against ischemic injury at 10 nM–1 μM [45]. Based on these reports, the effect of 0.1 nM–100 μM P was tested in the present study.

In conclusion, estrogens protected the cochlea against GM ototoxicity, especially βE2 affected through an ER-mediated pathway. DHEA also exerted protective effects, but its protective action was not concerned with ER. Given the growing evidence that estrogens and other neurosteroids are involved in many aspects of cochlear pathophysiology, it is important to clarify the precise mechanism of their actions in the cochlea.

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Protective Effects of Corticosteroids and Neurosteroids on Cochlear injury

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Abstract: Dysfunction of the cochlea causes sensorineural hearing loss. Glucocorticoids have been clinically applied for sensorineural hearing loss of sudden onset, including idiopathic sudden sensorineural hearing loss, acoustic injury, Meniere's disease, and immune-mediated hearing loss. However, clinical studies on sudden sensorineural hearing loss have revealed conflicting results regarding the efficacy of glucocorticoids. The findings obtained from animal experiments have demonstrated that glucocorticoids exhibited protective effects on some types of cochlear injury, but there were limitations regarding glucocorticoid therapy. Recently, the actions of neurosteroids in the cochlea have drawn much attention from auditory researchers. Clinical and experimental studies of the auditory system have indicated that estrogens affect auditory perception. Furthermore, estrogens and dehydroepiandrosterone (DHEA) exhibit protective effects on cochlear injury. This article was aimed to give an overview of steroid treatment for protection of the inner ear against various cochlear injuries. Findings obtained from animal studies are focused on.

Keywords: Cochlea, sensorineural hearing loss, glucocorticoid, neurosteroid, estrogen, steroid.

INTRODUCTION

Steroid hormones bind to intracellular receptors, and thereby regulate gene expression and protein synthesis. Mammalian steroid hormones can be divided into five groups based on the receptors to which they bind: corticosteroid hormones (glucocorticoid and mineralocorticoid hormones), androgen, estrogen, and progesterone. Most steroid hormones are secreted by the adrenal cortex, but some sex hormones are secreted by the testis or ovary.

Glucocorticoids have been used for the treatment of inner ear disorders such as idiopathic sudden sensorineural hearing loss, acoustic injury, acute labyrinthitis, and immune-mediated hearing loss. Although glucocorticoids have been used in the treatment of sensorineural hearing loss of sudden onset, clinical studies on sudden sensorineural hearing loss have revealed conflicting results regarding the efficacy of glucocorticoid therapy [1-3]. This is considered to be partially attributable to a variety of pathological conditions. The heterogeneity of the timing of glucocorticoid therapy may result in the heterogeneity of the treatment results. In addition, the precise protective mechanism of glucocorticoids has not yet been clarified.

Sex steroid hormones, including estrogen, progesterone, testosterone, and dehydroepiandrosterone (DHEA), are produced in the ovaries, testes, and adrenal glands. However, small amounts of these steroid hormones originate from non-endocrine tissue. Specifically, they can be synthesized de novo in the central nervous system from cholesterol, and are known as neurosteroids or neuroactive steroids. Recent studies have demonstrated that estrogens play important roles in maintaining the hearing function. The protective actions of estrogens and DHEA in the cochlea have also been reported.

In this review, we describe recent findings derived from animal experiments regarding steroid therapy for cochlear injuries.

GLUCOCORTICOID

Protective Effects of Glucocorticoid on Cochlear Injuries

Animal studies have demonstrated that glucocorticoids exhibit protective effects against various cochlear injuries. Prednisolone and methylprednisolone ameliorated ischemia-reperfusion injury of the cochlea in the guinea pig and chinchilla [4, 5]. These glucocorticoids protected cochlear sensory hair cells [6]. The protective effects were observed over a wide range of dosages of glucocorticoids when they were administered intravenously before the onset of ischemia [4]. The local application of dexamethasone to the round window niche also prevents cochlear damage caused by transient ischemia [7].

Glucocorticoids, including dexamethasone, prednisolone, and methylprednisolone, have protective effects against acoustic injury of the cochlea. They prevent the auditory threshold shift and hair cell death induced by acoustic overexposure under various treatment protocols [8-12].

The protective effects of glucocorticoids were also reported in cochlear injury induced by ototoxic agents. Although aminoglycosides are highly effective antibiotics against aerobic Gram-negative bacteria, these agents have well-known side effects, causing vestibular, cochlear, and renal toxicity. The clinical usage of aminoglycosides has often been limited because of their ototoxic side effects. Glucocorticoids reportedly protect hair cells against the oto-

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toxicity of aminoglycosides [13, 14]. Cisplatin is another representative ototoxic drug. It is a common anti-cancer agent used to treat many different types of cancer including esophageal, lung, uterus, and head and neck cancers. Animal experiments have demonstrated that glucocorticoids exert protective effects in this type of injury. Dexamethasone reduces auditory threshold shifts induced by high-dose cisplatin, and ameliorates the reduction in hair cell function after cisplatin therapy [15, 16].

Severe otitis media may induce labyrinthitis and cause cochlear dysfunction [17-20]. The damage is usually limited to the cochlear basal turn in this type of cochlear injury [21] because the round window membrane allows bacterial toxins to pass from the middle ear into the scala tympani of the cochlear basal turn. A disrupted round window membrane is thought to facilitate the passage of harmful substances from the middle to inner ear [22]. Experimental studies have demonstrated that glucocorticoids attenuate cochlear damage induced by labyrinthitis [21, 23].

Although glucocorticoids have been used in the treatment of sensorineural hearing loss, clinical studies have revealed conflicting results regarding the efficacy of glucocorticoid therapy [1-3]. This is considered to be partially attributable to the fact that sensorineural hearing loss of sudden onset includes a variety of pathological conditions. Furthermore, the heterogeneity of the timing of glucocorticoid therapy may result in the heterogeneity of the treatment results. The results of animal studies clearly demonstrated that glucocorticoids exhibited protective effects on some types of sensorineural hearing loss, as described above. Recent studies suggested that the timing of glucocorticoid treatment was extremely important to obtain optimal treatment results [24]. The protective effects of glucocorticoids seemed to decrease in post- as compared to pre-ischemic treatment [4]. In addition, the therapeutic time window of glucocorticoids is very short after the onset of acoustic injury [10]. These recent basic studies on cochlear injuries will advance our understanding of the effectiveness and limitations of glucocorticoids in the treatment of sensorineural hearing loss induced by various cochlear injuries.

Protective Mechanisms of Glucocorticoids in Cochlear Injuries

Two major isoforms of glucocorticoid receptors have been reported: glucocorticoid receptors α and β. It has been demonstrated that glucocorticoid receptor α is expressed in almost all cells and is fully functional after binding to glucocorticoids. On the other hand, glucocorticoid receptor β accounts for only 0.2-1.0% of total expression of glucocorticoid receptors and does not bind to glucocorticoids [25]. Immunohistochemical studies have demonstrated that glucocorticoid receptors are widely expressed in the cochlea [26, 27], although exact distribution of each glucocorticoid receptor has not been clarified. Namely, these glucocortioid receptors are abundantly expressed in the spiral ligament, and their expression has also been demonstrated in the other cochlear parts including the stria vascularis, organ of Corti, and spiral ganglion cells [26-28].

Glucocorticoids exert their actions *via* genomic and non-genomic pathways. The genomic actions are mediated by intracellular glucocorticoid receptors. The glucocorticoid receptors exist in cytosol consisting multiprotein complex with immunophilins, heat shock proteins, co-chaperones, and the mitogen-activated protein kinases. The binding of glucocorticoids to glucocorticoid recptors results in dissociation of the glucocorticoid receptors from the multiprotein complex. The glucocorticoid receptor then dimerizes and translocates into the nucleus [25]. The glucocorticoid/glucocorticoid-receptor complex induces or inhibits gene transcription by direct interactions with the glucocorticoid response elements (GREs) [29]. The non-genomic actions of glucocorticoids, on the other hand, may involve multiple mechanisms. Namely, such actions may be mediated by the non-specific interactions of glucocorticoids with cellular membranes, or mediated by intracellular and/or membrane-associated receptors. Recent studies have demonstrated using a glucocorticoid receptor antagonist, RU486, that glucocorticoids ameliorate cochlear injuries by binding to glucocorticoid receptors, at least in part [12, 30].

Generally, glucocorticoids inhibit the release of proinflammatory cytokines and stimulate the production of anti-inflammatory cytokines. Proinflammatory cytokines are also considered to be one of the important mediators of various cochlear injuries [31]. Glucocorticoids inhibit the synthesis and release of inflammatory cytokines in the cochlea [32]. Kalinec *et al.* [33] recently showed that glucocorticoids activate a myosin IIC-mediated mechanism that drives annexin A1 from the lipid droplets to the apical region of Hensen cells, where it is released into the external milieu. Their findings suggest that annexin A1 may be one of the major mediators of the anti-inflammatory effects of glucocorticoids in the cochlea. The inhibition of phospholipase A2 by glucocorticoids may mediate cochlear protection [12]. In addition, glucocorticoids enhance the synthesis of glutathione to decrease oxidative stress [34].

Nicolaides *et al.* [35] recently suggested that the effects of glucocorticoids on the immune system were mediated by protein-protein interactions, such as the interaction between the glucocorticoid receptor and nuclear factor kappa B, activator protein-1(AP-1), and signal transducers and activators of transcription (STATs). In addition to the classical genomic actions of glucocorticoids, they may protect the cochlea by affecting signal transduction cascades thorough protein-protein interactions. Tahera *et al.* [36] suggested that the glucocorticoid-induced translocation of nuclear factor kappa B from the cytosol into the nucleus was related to cell survival in acoustic injury.

Mineralocorticoids

Yao and Rarey [37] reported that mineralocorticoid receptors were expressed in the cochlea including inner and outer hair cells of the organ of Corti, stria vascularis, spiral ligament, spiral limbus, and spiral ganglion cells. Their expression was also demonstrated in the endolymphatic sac [38]. Although mineralocorticoid receptors are widely expressed in the cochlea, the precise action of mineralocorticoids remains unclear in cochlear pathophysiology. Turne *et al.* [39] reported that aldosterone was effective to preserve hearing level in MRL/MpJ-Fas^{lpr} autoimmune mice, and assumed that the protective effect was mediated by preserving the ion homeostatic functions of the cochlea. However, Mori

et al. [40] reported that the intravenous administration of aldosterone did not induce any change in the DC potential of the endolymphatic sac. Furthermore, Peters *et al.* [41] reported, based on human and animal data, that the mineralocorticoid aldosterone was not the crucial regulator of sodium transport in the cochlea.

Estrogen

17β-Estradiol (E2) is produced in the ovary, testis, and adrenal gland. Like other steroidal hormones, E2 enters the cell cytosol across the cell membrane and combines with an intra-nuclear receptor. The dimer (E2/estrogen receptor complex) activates target genes, which leads to the synthesis of corresponding mRNAs and proteins. The main physiological function of E2 is the sexual maturation of women, but recent reports have demonstrated that E2 also acts as a neurosteroid [42] and protects neurons against neuronal degeneration and death [43]. Besides the nervous system, E2 has protective effects against ischemic cardiovascular injury [44], renal failure [45], liver dysfunction [46], and oxidative damage to skeletal muscles [47]. Clinical and experimental studies have indicated that E2 plays significant roles in the auditory physiology [48]. Auditory perception changes during the menstrual cycle of women (Haggard and Gaston, 1978). Low-frequency tones and interaural time differences for sound localization were particularly susceptible to changes in the menstrual cycle [49]. Ovariectomy reportedly elevated auditory thresholds in rats [50]. Furthermore, otoacoustic emission, an indicator of outer hair cell function, is larger in females than males [51].

When the expression of estrogen receptors was investigated in the inner ears of rodents and humans, it was found that areas responsible for sound transmission (inner and outer hair cells and spiral ganglion cells) and inner ear homeostasis (stria vascularis and spiral ligament) were positive for estrogen receptors [52, 53]. The precise actions mediated *via* estrogen receptors α and β in the cochlea have not been clarified. In estrogen receptor β knockout mice, however, a rapid decline in hearing function after birth was observed and the mice became virtually deaf by one year of age [54]. The absence of hair cells and loss of the entire organ of Corti initiated in the basal turn of the cochlea were observed in the knockout mice. These findings suggest that estrogen receptor β is important for maintaining hair and spiral ganglion cells that are essential for the normal inner ear function.

Recent reports have demonstrated that estrogens have protective effects against cochlear injury. Meltser *et al.* [55] reported the significant elevation of ERβ protein expression in the cochlea of both male and female mice 24 h after acoustic trauma. They also reported that E2 had a protective effect against acoustic injury in mice, probably through binding to estrogen receptor β [55]. E2 also had protective effects against the gentamicin-induced damage of hair cells [56]. Recent findings have demonstrated that gentamicin-induced hair cell death occurs, at least in part, through an apoptotic process [57, 58]. An earlier report indicated that the c-jun N-terminal kinase (JNK) pathway was activated during the course of gentamicin-induced apoptosis and that gentamicin-induced hearing loss was alleviated by a JNK inhibitor [59, 60]. E2 treatment attenuated the activation of JNK and decreased the terminal deoxynucleotidyl tranferase-mediated biotinylated UTP nick-end labeling (TUNEL) of hair cells [56]. These findings suggest that E2 ameliorated the gentamicin-induced apoptotic death of hair cells through inhibition of the JNK pathway.

Dehydroepiandrosterone (DHEA)

DHEA is one of the most abundant neurosteroids, and is reportedly synthesized de novo in the nervous system [61]. It has been shown that DHEA acts as a neuromodulator by regulating the activity of gamma-aminobutyric acid (GABA) and N-methyl-D-aspartate (NMDA), and sigma 1 receptors [62, 63]. In addition, DHEA also exerts antioxidative actions [64]. It has been demonstrated that DHEA protects the cochlea against ischemia–reperfusion [4] and acoustic [65] injuries. DHEA also exhibits protective effects against gentamicin ototoxicity (Nakamagoe *et al.*, unpublished observation). When the cochlea is exposed to acoustic trauma or ischemia-reperfusion injury, free radical generation and glutamate excitotoxicity reportedly occur within it [66]. The precise protective mechanism of DHEA against cochlear injuries has not been clarified. However, it seems reasonable to suppose that it involves the same mechanism as reported in the central nervous system.

Progesterone and Testosterone

To date, the exact roles of progesterone and testosterone have not been made clear in cochlear pathophysiology. Although progesterone exhibits protective effects on neural cells in the brain [67], there is no report that progesterone protects the cochlea. Progesterone is used in conjunction with estrogen in hormone replacement therapy, and aged women undergoing hormone replacement therapy with progesterone and estrogen reportedly show poorer hearing abilities than those using estrogen alone [68]. Additionally, in peri-menopausal mice, hormone replacement therapy using both progesterone and estrogen accelerates age-related hearing loss compared to therapy utilizing estrogen alone [69]. Because the concurrent administration of estrogen and progesterone potently enhances prolactin release, these findings may be due to the bone dysmorphology of the otic capsule caused by hyperprolactinemia [70].

SUMMARY

The development of effective treatments for cochlear lesions and sensorineural hearing loss requires an understanding of the therapeutic agents as well as mechanisms of cochlear disease. Experimental studies have clearly established that glucocorticoids protect the cochlea against various cochlear injuries. Because of the difficulties associated with human clinical investigations, experimental animal studies offer opportunities to examine and explain the therapeutic mechanisms of glucocorticoid treatment. Recent findings of experimental animal studies have also demonstrated that treatment with neurosteroids, such as estrogens and DHEA, may be helpful for cochlear protection against various cochlear injuries. Of course, further studies are necessary to clarify the exact protective mechanisms of these steroid agents. An advance in our understanding of steroid therapy for cochlear lesions may improve the outcome of treatment for sensorineural hearing loss.

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ORIGINAL ARTICLE

Age-related hearing loss and expression of antioxidant enzymes in BDF1 mice

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Abstract

Conclusion. Our data suggest that the changes in expression of antioxidant enzymes may cause age-related hearing loss (AHL). *Objectives.* AHL is an aging process of the inner ear, and oxidant stressors are considered to be one of the leading causes. We investigated the hearing level and expression profile of antioxidant enzymes in aged mice. *Methods:* Mice aged 3, 6, and 11 months were used. Hearing levels of the mice were examined using the auditory brainstem response (ABR). After measuring the ABR threshold, cochleae were dissected. RNA was isolated from the cochleae, and cDNA was synthesized using the retro-transcription enzyme. Expression of the antioxidant enzymes was measured by quantitative real-time PCR. *Results:* The ABR thresholds of the BDF1 mice were elevated by 6 months of age. The expression of superoxide dismutase 1 (SOD1) and heme oxygenase 1 (HO1) at 11 months of age significantly decreased compared with that of those at 6 months of age. In contrast, a decrease in the expression level was not observed regarding NAD(P)H-quinone oxidoreductase 1 (NQO1).

Keywords: *Superoxide dismutase 1, SOD1, heme oxygenase 1, HO1, NAD(P)H-quinone oxidoreductase 1, NQO1*

Introduction

Age-related hearing loss (AHL) is the most common type of hearing disorder in aged subjects, and oxidant stressors are considered to be one of the leading causes of AHL in addition to genetic factors [1]. Living organisms have antioxidant systems that act against the oxidant stressors. McFadden et al. [2,3] reported that AHL was accelerated in superoxide dismutase 1 (SOD1) knockout mice. Based on their report, antioxidant enzymes may function to inhibit the progression of AHL. However, age-related changes in expression levels of antioxidant enzymes have not been clarified. We investigated the expression profile of the antioxidant enzymes in aged BDF1 mice. We examined three enzymes: heme oxygenase 1 (HO1), NAD(P)H-quinone oxidoreductase 1 (NQO1), and SOD1, in this study.

The selection of the mouse strain is important in conducting experiments regarding AHL because there are differences in age-related changes in the hearing level and cochlear morphology among strains. Many researchers used inbred mouse strains because they showed relatively uniform phenotypes. The C57Bl/6 mouse strain is considered to be one of the most favorable models for AHL [4]. On the other hand, Mikuriya et al. [5] recently showed that DBA/2 mice exhibited early-onset AHL, suggesting that the DBA/2 mouse strain was also suitable for examination of AHL. BDF1 mice are hybrids of C57Bl/6 and DBA/2 strains. The merit of using this hybrid strain is that it is relatively healthy even when

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aged. We also examined age-related changes in hearing levels and the cochlear morphology in BDF1 mice in this study.

Material and methods

Animals

C57Bl/6 and BDF1 mice were obtained from CLEA Japan Inc. (Tokyo, Japan). The animals were maintained under a normal day/night cycle and had free access to food and water. The care and use of animals were approved by the Animal Research Committee and Safety Committee for Gene Recombination Research of the University of Tsukuba.

Measurement of auditory brainstem response (ABR)

Mice were anesthetized by intraperitoneal injection of 50 mg/kg pentobarbital sodium. Positive, negative, and ground electrodes were inserted subcutaneously at the vertex, mastoid, and back, respectively [6,7]. ABR responses were evoked by tone bursts of pure tones at 4, 8, and 16 kHz (rise and fall times, 1 ms; duration, 10 ms). Evoked responses were filtered with a band pass of 200–3000 Hz and averaged with 1000 sweeps using a signal processor (Synax 2100, NEC, Tokyo, Japan). The sound intensity was varied in 5 dB steps. The hearing threshold was defined as the lowest intensity yielding the consistent appearance of at least one ABR peak.

Evaluation of hair cell loss and spiral ganglion cell count

The mice were sacrificed under deep anesthesia. Cardiac perfusion was performed with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS). Cochleae were then removed and immersed in the same fixative for 8 h at 4°C. After fixation, the cochleae were decalcified with ethylenediamine tetraacetic acid (EDTA). Whole mounts of the organ of Corti were permeabilized with 5% Triton X-100 (Sigma, St Louis, MO, USA) in PBS with 10% fetal bovine serum for 10 min. The specimens were stained with a rhodamine-phalloidin probe (1:100, Invitrogen, Carlsbad, USA) at room temperature for 1 h [8]. Phalloidin is a specific marker for cellular F-actin and labels stereociliary arrays and cuticular plate of hair cells. All specimens were thoroughly inspected under a laser confocal microscope from the apex to base (TCS 4D, Leica Microsystems, Wetzlar, Germany). Quantitative results were obtained by evaluating 30 outer hair cells associated with 10 inner hair cells in a given microscope field in each turn. The average of three separate counts was used to represent each specimen. For counting spiral ganglion cells, the fixed and decalcified cochleae were embedded in paraffin. The cochleae were cut into midmodiolar sections of 5 μm thickness. After the sections were stained with hematoxylin and eosin, the numbers of spiral ganglion cells were counted.

Reverse transcription-polymerase chain reaction (RT-PCR)

BDF1 mice were sacrificed under deep anesthesia. Cochleae were dissected under a microscope, and the organ of Corti was removed for the RT-PCR study. Total RNAs were isolated from the culture specimens using an Isogen RNA preparation kit (Nippon Gene, Tokyo, Japan). The cDNA samples were synthesized with Superscript II reverse transcriptase (Invitrogen). Real-time RT-PCR was performed to quantify mRNA levels of HO1, NQO1, and SOD1 using an ABI-PRISM 7700 Sequence Detector System (Applied Biosystems, Carlsbad, USA). For analyses of HO1 and NQO1, primers and TaqMan probes were added to the master mixture that contained all reagents for PCR (Perkin-Elmer, Waltham, USA). For analysis of SOD1, the primers (forward: 5's-GACAAACCTGAGCCCTAAG-3's; reverse: 5's-CGACCTTGCTCCTTATTG-3's) were added to the Power SYBR Green RNA-to-CT 1 step kit (Applied Biosystems).

Data analysis

All data are presented as means ± SEM. ABR threshold shifts and missing hair cell rates were evaluated with two-way ANOVA using StatView 5.0. A p value of < 0.05 was considered significant.

Results

ABR thresholds of aged BDF1 mice and comparison with those of C57BL/6J mice

Hearing thresholds of BDF1 mice were examined using ABR (Figure 1). The ABR thresholds were elevated at 6 months of age as compared with those at 3 months ($n = 10$ in each group, two-way ANOVA and the Bonferroni test: $^{**}p < 0.01$). ABR thresholds further increased at 11 months ($n = 10$ in each group, two-way ANOVA and the Bonferroni test: $^{**}p < 0.01$ compared with 6 months). In addition to the BDF1 mice, we examined ABR thresholds of C57Bl/6 mice. C57Bl/6 mice also showed an elevation of ABR thresholds on aging. Namely, ABR thresholds were significantly higher at 11 than at 6 months (two-way ANOVA and the Bonferroni test: $^{**}p < 0.01$).

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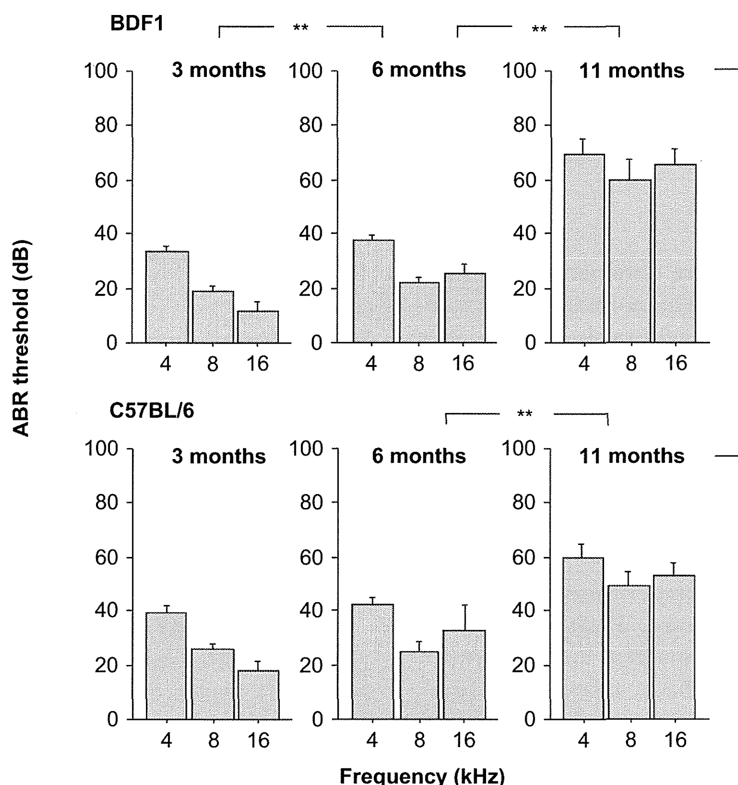

Figure 1. ABR thresholds of BDF1 and C57Bl/6 mice. ABR thresholds were examined at 3, 6, and 11 months of age in both mouse strains. In BDF1 mice, ABR thresholds were elevated at 6 months as compared with those at 3 months, and those at 11 months were significantly higher than at 6 months (two-way ANOVA: $**p < 0.01$). In C57Bl/6 mice, ABR thresholds were elevated by 11 months (two-way ANOVA: $**p < 0.01$). When comparing those two mouse strains, ABR thresholds were higher in BDF1 than in C57Bl/6 mice (two-way ANOVA: $*p < 0.05$).

ABR thresholds of BDF1 mice were significantly higher than those of C57Bl/6 mice at 11 months ($n = 10$ in each group).

Loss of hair cells and spiral ganglion cells in aged BDF1 mice

Hair cell loss of each turn of the cochlea was examined using phalloidin-stained surface preparation specimens at 3, 6, and 11 months in BDF1 mice. A significant loss of outer hair cells was observed at 6 months (Figure 2B, two-way ANOVA and the Bonferroni test: $**p < 0.01$). Hair cell loss became more evident by 11 months. On the other hand, the loss of inner hair cells at 6 months was almost the same as that at 3 months. Significant inner hair cell loss was observed by 11 months of age (Figure 2C). There was a tendency whereby more cell loss was observed in the lower than the upper turns, and in the outer than inner hair cells.

The number of spiral ganglion cells was counted using midmodiolar sections at 3, 6, and 11 months of age. Spiral ganglion cells decreased by 11 months of age (Figure 3).

Expression of mRNAs of antioxidant enzymes

The mRNA expression of *Ho1*, *Nqo1*, and *Sod1* relative to *GAPDH* was examined at 6 ($n = 5$) and 11 ($n = 5$) months. As shown in Figure 4, the expression level of *Nqo1* mRNA did not change on aging. However, mRNA expression of *Ho1* and *Sod1* significantly decreased at 11 months as compared with 6 months (one-way ANOVA: $p < 0.05$ for expression of *Ho1* and *Sod1*).

Discussion

BDF1 mice were created as a hybrid strain of C57Bl/6 and DBA/2 mice. The BDF1 strain is thus closely related to the C57Bl/6 strain, which rapidly develops AHL [9]. We compared ABR thresholds between the BDF1 and C57Bl/6 mice as a function of age. The BDF1 mice exhibited significant AHL by 6 months. ABR thresholds were elevated in the BDF1 as compared with the C57Bl/6 mice at 11 months of age, showing that BDF1 mice exhibited more severe AHL than C57Bl/6 mice (Figure 1). By this age, BDF1 mice showed the loss of inner and outer hair cells and spiral ganglion cells (Figures 2 and 3).

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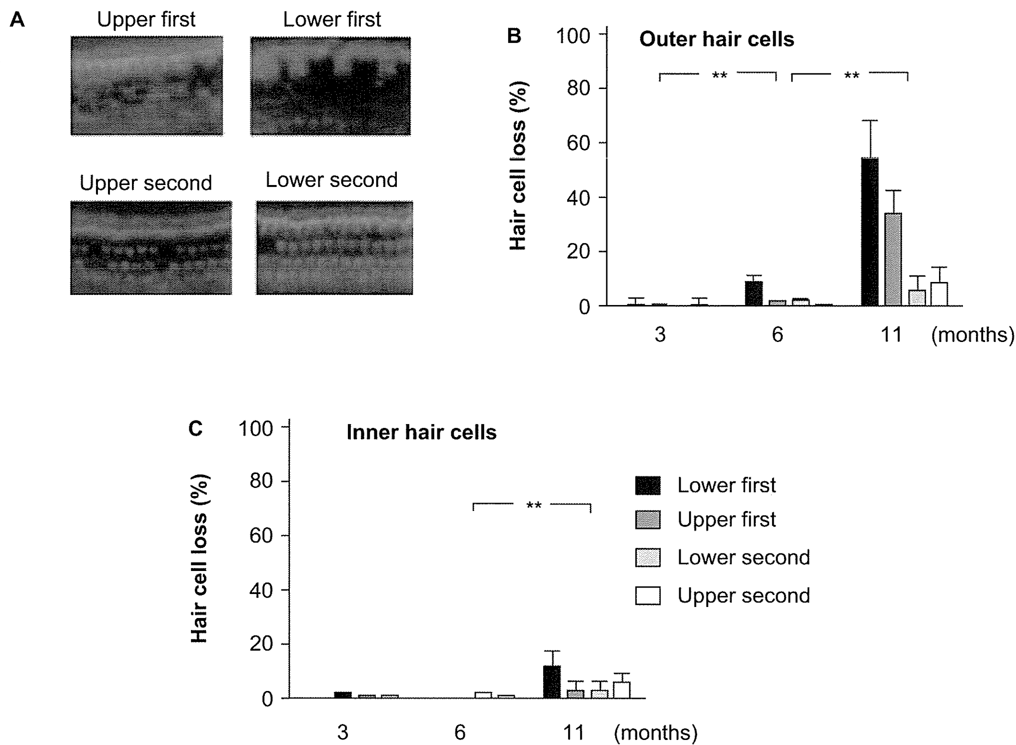

Figure 2. Hair cell loss in BDF1 mice. (A) Representative photographs of phalloidin-stained hair cells at 11 months. Hair cell loss was evident in the upper turns. (B and C) Quantitative analysis of inner and outer hair cell loss. Hair cell loss was observed at 6 months in outer hair cells, and at 11 months in inner hair cells (two-way ANOVA: $**p < 0.01$).

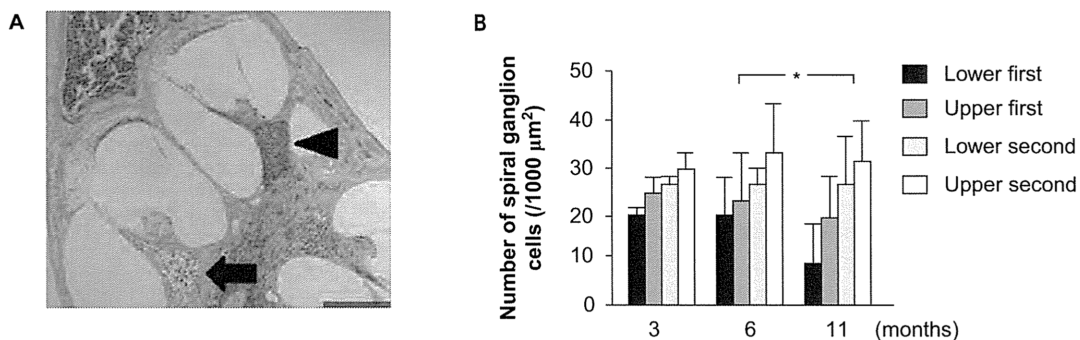

Figure 3. Spiral ganglion cells. (A) Representative photograph of spiral ganglion cells at 11 months; arrow, upper second turn; arrowhead, upper first turn. (B) Quantitative analysis of spiral ganglion cells. Spiral ganglion cells decreased by 11 months (two-way ANOVA: $*p < 0.05$).

The present PCR findings demonstrated that expression levels of antioxidant enzymes changed during aging. SOD1, dismutasing superoxide to hydrogen peroxide, is one of the best characterized enzymes in cochlear pathology. For example, AHL was accelerated in SOD-deficient mice, and SOD-deficient mice were demonstrated to be vulnerable to cochlear acoustic injury [2,3,10]. Tempol, an SOD mimetic agent, also alleviated acoustic injury [7]. In addition to SOD1, antioxidant properties of HO1 were also reported. HO is a rate-limiting enzyme in heme catabolism, which leads to the generation of biliverdin, free iron, and carbon monoxide [11]. Three HO isoforms have been identified, as well as HO1 responses to various oxidative stresses. Regarding cochlear pathology, the expression of HO1 was reportedly induced by intense noise exposure [12]. The pharmacological induction of HO1 suppressed the apoptosis of cochlear HEI-OC1 cells induced by cisplatin [13]. These results indicate that SOD1 and HO1 may serve as safeguards of auditory hair cells against oxidative stress induced by various cochlear injuries. There is a possibility, therefore, that decreases in expression levels of SOD1 and HO1 may accelerate the hair cell loss observed in AHL.

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