Fenofibrate exerts protective effects against gentamicin-induced toxicity in cochlear hair cells by activating antioxidant enzymes

CHANNY PARK[1-3*], HYE-MIN JI[1*], SE-JIN KIM[1,5], SUNG-HEE KIL[2], JOON NO LEE[1,5], SEONGAE KWAK[4], SEONG-KYU CHOE[1] and RAEKIL PARK[5]

[1]Department of Microbiology and Center for Metabolic Function Regulation, Wonkwang University School of Medicine, Iksan, Jeonbuk 54538, Republic of Korea; [2]Division of Cell Biology and Genetics, House Research Institute, Los Angeles, CA 90057, USA; [3]Department of Physiology, Seonam University School of Medicine, Namwon, Jeonbuk 55724; [4]Zoonosis Research Center, Wonkwang University School of Medicine, Iksan, Jeonbuk 54538; [5]Department of Biomedical Science and Engineering, Gwangju Institute of Science and Technology, Gwangju 61005, Republic of Korea

Received April 4, 2016; Accepted February 24, 2017

DOI: 10.3892/ijmm.2017.2916

Abstract. Fenofibrate, an activator of peroxisome proliferator-activated receptors (PPARs), has been shown to protect the kidneys and brain cells from oxidative stress; however, its role in preventing hearing loss has not been reported to date, at least to the best of our knowledge. In this study, we demonstrated the protective effects of fenofibrate against gentamicin (GM)-induced ototoxicity. We found that the auditory brainstem response threshold which was increased by GM was significantly reduced by pre-treatment with fenofibrate in rats. In cochlear explants, the disruption of hair cell layers by GM was also markedly attenuated by pre-treatment with fenofibrate. In addition, fenofibrate almost completely abolished GM-induced reactive oxygen species generation, which seemed to be mediated at least in part by the restoration of the expression of PPAR-α-dependent antioxidant enzymes, including catalase and superoxide dismutase (SOD)-1. Of note, fenofibrate markedly increased the expression of heme oxygenase-1 (HO-1) which was also induced to a certain degree by GM alone. The induced expression of HO-1 by fenofibrate appeared to be essential for mediating the protective effects of fenofibrate, as the inhibition of HO-1 activity significantly diminished the protective effects of fenofibrate against the GM-mediated death of sensory hair cells in cochlea explant culture, as well as in zebrafish neuromasts. These results suggest that fenofibrate protects sensory hair cells from GM-induced toxicity by upregulating PPAR-α-dependent antioxidant enzymes, including HO-1. Our results provide insight into the preventive therapy for hearing loss caused by aminoglycoside antibiotics.

Correspondence to: Dr Seong-Kyu Choe, Department of Microbiology, Wonkwang University School of Medicine, 460 Iksandae-ro, Iksan, Jeonbuk 54538, Republic of Korea
E-mail: seongkyu642@wku.ac.kr

Dr Raekil Park, Department of Biomedical Science and Engineering, Gwangju Institute of Science and Technology, 123 Cheomdangwagi-ro, Gwangju 61005, Republic of Korea
E-mail: rkpark@gist.ac.kr

*Contributed equally

Key words: gentamicin, fenofibrate, protective effects, heme oxygenase, ototoxicity, zebrafish lateral line

Introduction

The adverse effects of aminoglycosides prominently target the kidneys, vestibular and auditory organs, and neuromuscular junction. Nephrotoxicity is reversible and can be clinically managed with hydration therapy so that patients generally recover normal renal function once treatment with aminoglycosides is discontinued (1). By contrast, ototoxicity may be initially overlooked, as it can occur after the end of drug treatment and develops only slowly thereafter. Gentamicin (GM), an aminoglycoside antibiotic, is clinically used in the treatment of infectious diseases caused by Gram-negative or Gram-positive organisms, including *Pseudomonas*, *Proteus*, *Serratia* and *Staphylococcus* species (2), as well as severe diseases, such as Meniere's disease and tuberculosis (3). The incidence of hearing loss ranges from a very low percentages up to 33%, and vestibular toxicity occurs in approximately 15% of patients who receive aminoglycoside antibiotics (4). However, GM remains widely used in developing countries as it is cost-effective and not subject to strict regulations by prescription. Therefore, developing otoprotective strategies is a primary and urgent goal for the prevention of GM-induced ototoxicity.

GM-induced cell death is thought be mediated by reactive oxygen species (ROS) (5-9), and several agents that scavenge ROS or block their formation have been proposed to protect the inner ears (10-14). To protect against the destructive effects of ROS, living cells have developed various defense systems, including enzymatic antioxidants, such as catalase, superoxide dismutases (SODs), glutathione peroxidase and heme oxygenase-1 (HO-1). Particularly, $O_2^{\bullet-}$ is converted to less reactive H_2O_2 and O_2 by SODs, and H_2O_2 is further converted to

H_2O and O_2 by either the catalase located in the peroxisomes or by glutathione peroxidase located in the mitochondria and cytoplasm (15). The enhanced expression of SOD-1 has been shown to exert protective effects against diverse types of tissue injury, such as ischemic and reperfusion injury, hypoxic lung injury, brain trauma, various chemicals and drugs (16-19). Similarly, HO-1 induced by various oxidative agents as a stress-responsive protein plays versatile roles in the protection of cells from various oxidative stresses (20-23).

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors that belong to the nuclear receptor superfamily (24). The activation of PPARs by their ligands reduces inflammation by decreasing cytokines, adhesion molecules and nitric oxide synthase 2, and reduces oxidative stress by increasing antioxidant enzymes in different experimental models (25-30). PPAR-α, a member of the PPAR family, plays a critical role in important physiological processes, such as the regulation of lipoproteins, lipid metabolism and glucose homeostasis, and has been implicated in relieving oxidative stress (31). Accordingly, a PPAR-α-specific binding site was identified in the promoter regions of catalase and SOD-1, suggesting that PPAR-α may directly regulate the expression of these genes (32). Recently, fenofibrate, a PPAR-α agonist that belongs to the fibrate class, has been shown to protect the kidneys by suppressing oxidative stress (33); however, its otoprotective effects against ROS have not been reported to date, at least to the best of our knowledge. In this study, we investigated the protective effects of fenofibrate on the GM-induced death of sensory hair cells in both cochlea explant cultures of rats, and in an *in vivo* zebrafish model.

Materials and methods

Reagents. Fenofibrate, GM, tin protoporphyrin IX (SnPPIX), phalloidin-tetramethylrhodamine isothiocyanate (TRITC), Triton X-100 and gelatin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Plastic culture dishes were obtained from BD Biosciences (Franklin Lakes, NJ, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), 2',7'-dichlorofluorescein diacetate (DCFH-DA) and Yo-Pro1 were obtained all from Invitrogen Life Technologies (Carlsbad, CA, USA). Antibodies, including anti-PPAR-α (sc-1985), anti-catalase (sc-34285), anti-SOD-1 (sc-11407), anti-HO-1 (sc-1796) and anti-β-actin (sc-47778), were purchased all from Santa Cruz Biotechnology, Inc., (Santa Cruz, CA, USA).

Animals. Sprague-Dawley (SD) rats (n=30, 15 male and 15 female) were purchased from Orient Bio, Inc. (Gyeonggi-do, Korea). The SD rats were fed a standard commercial diet and were housed at an ambient temperature of 20-22˚C and relative humidity of 50±5% under a 12-h light/12-h dark cycle in a specific pathogen-free facility. Experiments were performed using in-house born 3-week-old SD rats weighing between 30 and 35 g, and all rats were age-matched to within 3 days. For each experiment, 40 rats were divided into 4 different treatment groups (n=10 per group): saline group (control), intraperitoneally-injected with 200 mg/kg GM for 4 days (GM), 100 mg/kg fenofibrate (FF) for 10 days followed by GM (GM + FF), and fenofibrate alone (FF). At the end of the treatment, the rats were anesthetized for measuring auditory brainstem response (ABR) and sacrificed for conducting immunohistochemistry to detect expression of antioxidant enzymes. Zebrafish (*Danio rerio*) were bred through the paired mating of wild-type fish. They were maintained at 28.5˚C on a 14-h light/10-h dark cycle. Zebrafish were tested at the 5th day post-fertilization and maintained in an incubator at 28.5˚C during treatment. Larvae were immersed in a Petri dish containing 15 ml embryo medium (EM) [13.7 mM NaCl, 540 μM KCl (pH 7.4), 25 μM Na_2HPO_4, 44 μM KH_2PO_4, 300 μM $CaCl_2$, 100 μM $MgSO_4$ and 420 μM $NaHCO_3$ (pH 7.4)]. Larvae were treated with fenofibrate-containing EM for 30 min prior to the addition of GM (in EM) for 1 h, and then rinsed 4 times in EM. Hair cell survival was assessed by Yo-Pro1 labeling. All animal experiments were approved by the Institutional Animal Care and Use Committee at Wonkwang University (WKU16-2; Iksan, Korea).

Organotypic cultures of Corti organ explants. For *ex vivo* explant cultures, we sacrificed in-house born rats on post-natal day 3, and the temporal bones were isolated in a sterile manner. After placing the tissue in a 6-cm dish with ice-cold phosphate-buffered saline (PBS; pH 7.4), the cochlear capsule was peeled away and the membranous labyrinth was exposed. The spiral ligament and stria vascularis (SV) were removed and the organ of Corti was dissected under a microscope. Each explant was placed onto a 0.1% gelatin-coated glass coverslip in a 4-well dish containing DMEM supplemented with 10% FBS. Culture wells, each containing 500 μl of medium, were maintained in an incubator at 37˚C for 16 h with 5% CO_2 and 95% humidity. For each experiment, four 3-day-old rats were equally divided into 4 different treatment groups (n=2 ears per group): control, GM, GM + FF, and FF.

Phalloidin staining. The cochlear explants were fixed with 4% paraformaldehyde in PBS at room temperature for 15 min, washed with PBS (pH 7.4), and incubated with 0.1% Triton X-100 at room temperature for 15 min. The explants were stained with TRITC-labeled phalloidin (1:1,000) in PBS for 30 min in the dark, and washed 3 times with PBS. The cochlear explants were observed using a fluorescence microscope (IX71; Olympus, Tokyo, Japan) equipped with a digital camera (DP70; Olympus, Tokyo, Japan). Morphologically, intact hair cells were counted in a section corresponding to 10 inner hair cells at three different zones located on the basal turn of explants.

Measurement of intracellular ROS levels. Intracellular ROS levels were measured using the fluorescent dye, DCFH-DA. In the presence of an oxidant, DCFH-DA is converted into highly fluorescent 2',7'-dichlorofluorescein (DCF). The cochlear explants were pre-treated with 100 μM fenofibrate for 4 h and then exposed to 300 μM GM for 12 h. Following incubation, the samples were incubated with 10 μM DCFH-DA for 30 min. The fluorescence was detected under a fluorescence microscope. A microplate reader was used to quantify the ROS levels. Cochlear explants were plated in 96-well plates overnight and were pre-treated with 100 μM fenofibrate for 4 h and then exposed to 300 μM GM for 12 h. After washing with PBS, serum-free DMEM containing 10 μM DCFH-DA was added to each well and the plates were incubated at 37˚C for 1 h. ROS production was measured using a microplate reader equipped with a spectrofluorometer (SpectraMax M3; Molecular Devices,

Sunnyvale, CA, USA) at an emission wavelength of 538 nm and an excitation wavelength of 485 nm. Relative ROS production was expressed as the change in fluorescence of experimental groups compared with that of the appropriate controls (100%).

Western blot analysis. Each sample consisted of an apex, middle and base. The explants were collected from the media, washed with ice-cold PBS, centrifuged at 3,000 rpm for 3 min at 4˚C, lysed with 30 μl lysis buffer [50 mM Tris, pH 6.8, 10% glycerol, 2% sodium dodecyl sulfate (SDS), 0.005% bromophenol blue, 100 mM dithiothreitol, 1 mM sodium fluoride, 1 mM sodium orthovanadate, 1X proteinase inhibitor and 1 mM phenylmethylsulfonyl fluoride], and boiled for 15 min to denature the proteins. Following centrifugation at 13,000 rpm for 10 min at 4˚C, the supernatants were collected and loaded for SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Equal volumes (15 μl) of these supernatants were separated by 10% SDS-PAGE, and electrotransferred onto nitrocellulose membranes. The nitrocellulose membranes were then blocked with 5% non-fat dried milk in TBS-T (50 mM Tris-HCl, pH 7.4, 150 mM NaCl and 0.1% Tween-20) for 60 min at room temperature. The blots were incubated overnight at 4˚C with primary antibodies (1:1,000) in 3% non-fat dried milk in TBS-T, washed extensively with TBS-T, and incubated with a horseradish peroxidase (HRP)-conjugated anti-rabbit (A120-101p; Bethyl Laboratories, Montgomery, TX, USA) or anti-goat (P0449; DAKO, Glostrup, Denmark) IgG antibody (1:2,000) for 1 h. The immunoreactive signal was detected using an enhanced chemiluminescence detection system. To quantify band intensity, the images of immunoblot films were scanned; band intensity was quantified using the Gel-Pro Analyzer 4.0 software program and presented as the indicated ratio compared to the control expression level (expression level of the control was regarded as 1-fold). The protein expression levels of each enzyme were normalized to those of β-actin.

Yo-Pro1 staining. To assess ototoxicity, 5-day-old zebrafish were treated with GM added directly to the EM. Twenty embryos were used for each treatment. Additionally, at 5 days post-fertilization, zebrafish larvae were exposed to either 50 μM GM, 10 μM fenofibrate and 50 μM GM, 10 μM fenofibrate, or SnPP, fenofibrate and GM. The hair cell lateral line neuromasts were labeled with 2.5 μM Yo-Pro1 (Molecular Probes, Eugene, OR, USA) for 30 min, followed by washing 3 times. The zebrafish were then rinsed 3 times (5 min/wash) with EM and anesthetized with 8 μg/ml MS-222 (Sigma-Aldrich). The zebrafish were mounted with methylcellulose on a depression slide for observation under a fluorescence microscope.

Auditory brainstem response. Auditory brainstem response (ABR) was measured using System 3 hardware and software (Tucker Davis Technologies, Alachua, FL, USA), with 1,000 stimulus repetitions/record. Three-week-old SD rats were anesthetized using a mixture of ketamine (40 mg/kg) and xylazine (10 mg/kg) and kept warm with a heating pad during ABR recording. A subdermal needle electrode was inserted at the vertex, while ground and reference electrodes were inserted subdermally into the loose skin beneath the pinnae of opposite ears. Tone bursts of 4-msec durations and a rise-fall time of 1 msec at frequencies of 4, 8, 16 and 32 kHz were presented to the right ear and left ear through an insert speculum in the external auditory meatus. Sound intensity was varied at 5-dB intervals near the threshold. Judgment of the threshold was made off-line, based on the ABR records, by two independent, experimentally blinded observers.

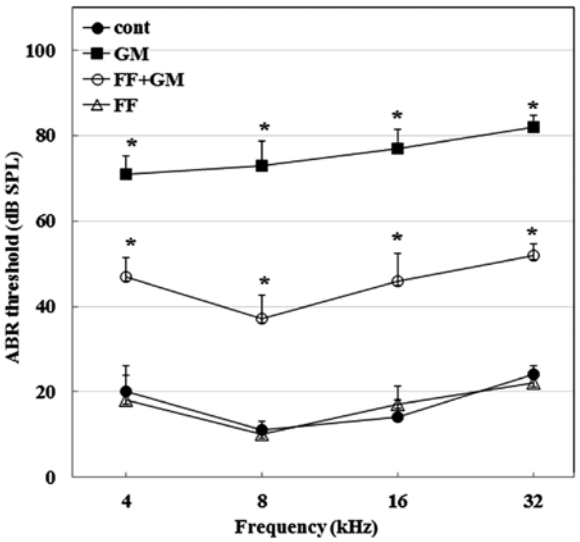

Figure 1. Effect of fenofibrate on GM-induced hearing loss in adult rats. ABR thresholds were measured at 4, 8, 16 and 32 kHz in the four groups: saline-injected, GM, GM + FF and FF groups. Average ABR thresholds were calculated from ABR recordings of 10 ears in each group. Mean ABR thresholds were plotted in response to tone-burst stimuli at each frequency. *p<0.001 by one-way ANOVA. FF, fenofibrate; GM, gentamicin; ABR, auditory brainstem response.

Cochlea immunohistochemical analysis. For immunohistochemical analysis, a Dako immunohistochemistry kit (LSAB Universal K680; Dako, Carpenteria, CA, USA) was used according to the manufacturer's instructions. The removed temporal bone was fixed in 4% paraformaldehyde for 16 h, and then decalcified with 10% EDTA in PBS for 2 weeks, dehydrated, and embedded in paraffin wax. Sections (4-μm-thick) were deparaffinized in xylene and rehydrated in increasing ethanol concentrations. Endogenous peroxidase was blocked with 3% hydrogen peroxide for 5 min at room temperature following PBS washing. Non-specific binding was blocked with 1% bovine serum albumin (BSA) for 1 h. Subsequently, each antibody was added to the slides and incubated for 1 h. Following repeated washes with PBS, the sections were incubated with a biotinylated secondary antibody in the kit for 1 h and covered for 30 min with streptavidin-peroxidase. Finally, the sections were stained in freshly prepared substrate solution (3 mg of 3-amino-9-ethylcarbazole in 10 ml of sodium acetate buffer pH 4.9, 500 μl of dimethylformamide, 0.03% hydrogen peroxide) for 10 min. The nuclei of immunostained cells were counterstained with Mayer's hematoxylin (Sigma-Aldrich).

Statistical analysis. Each experiment was performed independently at least 3 times, and all values represent the means ± standard deviation (SD) of triplicate experiments. One-way analysis of variance (ANOVA) was used to analyze the statistical significance of the results. Reported error bars are one SD from the mean. p-values ≤0.005 were considered to indicate statistically significant differences.

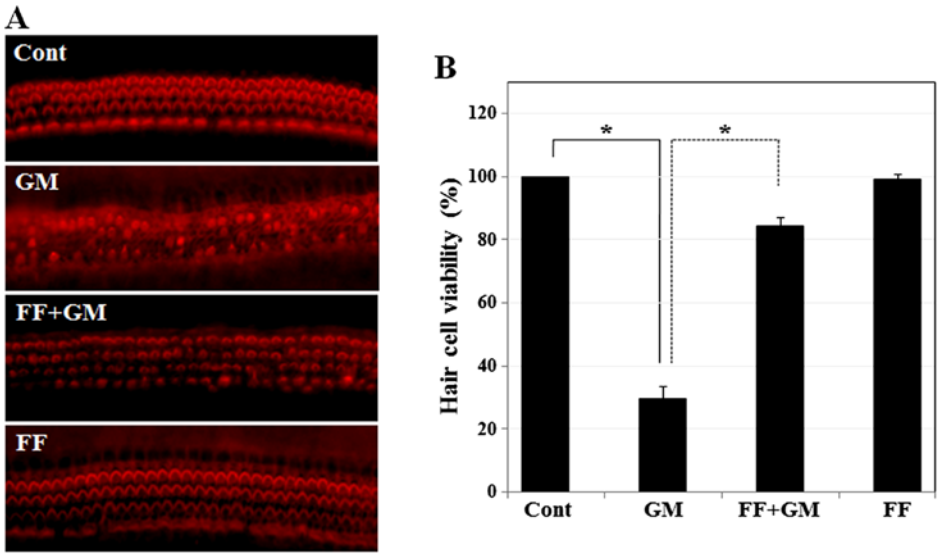

Figure 2. Effect of fenofibrate on GM-induced hair cell death in rat cochlear explants. (A) Hair cells were stained with phalloidin-TRITC and observed under a fluorescence microscope. Cochlear explants were treated with medium alone, GM (300 μM) for 24 h (29.6±3.78, p≤0.00002), fenofibrate (100 μM) pre-treated for 4 h and then co-treated with GM (300 μM) for 24 h (84.4±2.44, p≤0.0005), or fenofibrate (100 μM) only for 28 h (99.3±1.33, NS). (B) Quantitative analysis of survival of the sensory hair cells. Histogram shows the mean viability of the sensory hair cells. The data represent the means ± SD of 3 independent experiments. *p<0.001 by one-way ANOVA, compared with the control or GM-treated group. GM, gentamicin; FF, fenofibrate; TRITC, phalloidin-tetramethylrhodamine isothiocyanate.

Results

Fenofibrate prevents GM-induced hearing loss in rats. To examine the preventive effects of fenofibrate against GM-induced hearing loss *in vivo*, we first compared the ABR thresholds of four different treatment groups: saline group (control), intraperitoneally injected with 200 mg/kg GM for 4 days (GM group), 100 mg/kg fenofibrate for 10 days followed by GM (GM + fenofibrate group) and fenofibrate alone (FF group). As shown in Fig. 1, at the end of drug treatment on day 14, the average ABR thresholds at all frequencies in the GM group were significantly higher than those in the control group (p≤0.0005, n=10), confirming that GM induces hearing loss in rats. However, the administration of fenofibrate (GM + fenofibrate group) significantly reduced tone burst ABR as compared to GM alone. Fenofibrate alone did not affect hearing sensitivity. Therefore, these results indicate that GM causes hearing loss, which may be prevented by the use of fenofibrate in rats.

Pre-treatment with fenofibrate protects sensory hair cells of rat cochlear explants from GM-induced toxicity. Since GM induces hearing loss by disrupting sensory hair cells, we then investigated whether fenofibrate protects sensory hair cells from GM-induced toxicity in an organotypic culture of cochlear explants isolated from SD rats at post-natal day 3. In the control group, sensory hair cells visualized by TRITC-conjugated phalloidin appeared as three rows of outer hair cells and a single row of inner hair cells (Fig. 2A). However, exposure to GM resulted in the destruction of stereocilia bundles and induced a disordered array of hair cells. By contrast, pre-treatment with fenofibrate protected the sensory hair cells from the damaging effects of GM, displaying a well-preserved pattern of layers in outer hair cells and inner hair cells. Fenofibrate alone did not induce damage to the cochlear hair cells. We also quantified the number of cells that survived after each set of drug treatment in the cochlear explants. We observed a significant reduction in the survival rate (%) of sensory hair cells following exposure to GM (Fig. 2B). However, the survival rate of sensory hair cells in the rat cochlear explants pre-treated with fenofibrate was significantly higher than that in the GM-exposed explants. Taken together, our results indicate that fenofibrate protects auditory hair cells from GM-induced cell death.

Fenofibrate reduces GM-induced oxidative stress in rat cochlear explants. Since the overproduction of ROS is a major cause of GM-induced sensory hair cell death (34-37), the protective effects of fenofibrate may be mediated by reducing the ROS levels induced by GM. We thus quantified the ROS levels in cochlear explants by staining with DCFH-DA (DCF), a fluorescent probe for measuring intracellular ROS production. As shown in Fig. 3, GM induced a strong DCF signal, whereas pre-treatment with fenofibrate significantly reduced the DCF intensity to a level almost comparable to that of the control, indicating that fenofibrate prevents GM-induced oxidative stress. Fenofibrate alone slightly decreased the ROS levels, suggesting that the drug itself has an antioxidant effect.

Fenofibrate increases the expression of antioxidant enzymes in rat cochlear explants. Previously, it has been shown that the activation of PPAR-α by fenofibrate exerts a protective effect against oxidative stress in the kidneys (38). In addition, PPAR-α is known to regulate its own expression (39). To examine whether the otoprotective effects of fenofibrate are mediated by the regulation of PPAR-α and antioxidant enzymes, we measured the expression levels of PPAR-α and antioxidant proteins. Consistent with the protective effect of fenofibrate previously observed in the kidneys, pre-treatment with fenofibrate restored the expression of catalase, SOD-1 and PPAR-α, the levels of which were all significantly reduced

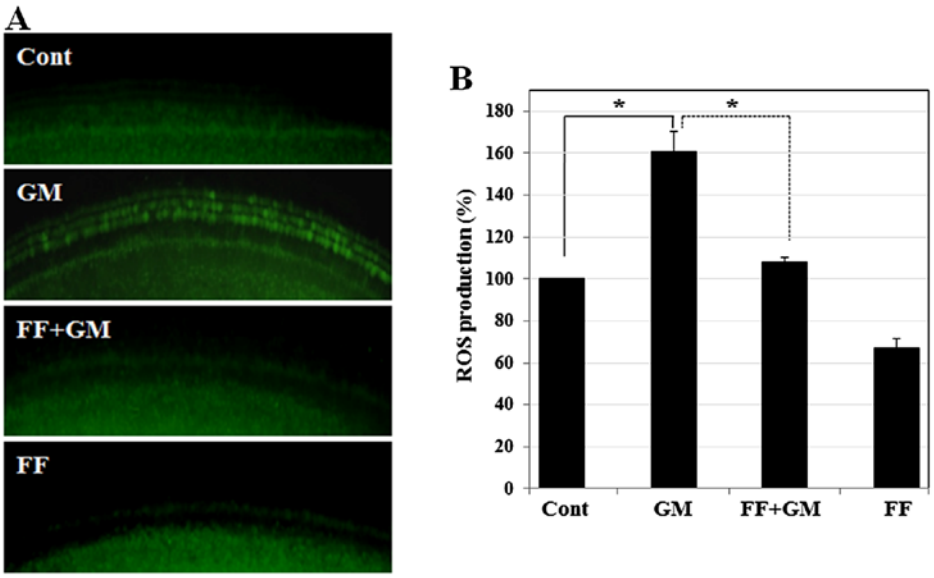

Figure 3. Effect of fenofibrate on GM-induced oxidative stress in rat cochlear explants. (A) Intracellular ROS levels in the sensory hair cells were monitored using DCFH-DA under a fluorescence microscope. Cochlear explants were treated with medium alone, GM (300 μM) for 12 h (161±9.2, p≤0.0004), fenofibrate (100 μM) pre-treated for 4 h and then co-treated with GM (300 μM) for 12 h (108±2.3, p≤0.0004), and fenofibrate (100 μM) only for 16 h (67±4.3, p≤0.0002). (B) Intracellular ROS levels in the sensory hair cells were determined using DCFH-DA under a microplate reader. The histogram shows the mean ROS production. *p<0.001 by one-way ANOVA, compared with the control or GM-treated group. GM, gentamicin; FF, fenofibrate; ROS, reactive oxygen species; DCFH-DA, 2',7'-dichlorofluorescein diacetate.

by GM (Fig. 4A). However, the expression of HO-1 seemed to be regulated differently from the other antioxidant enzymes. In particular, we found that GM significantly increased the expression of HO-1, which was barely detectable in the controls. In addition, either pre-treatment with fenofibrate or fenofibrate alone further induced the level of HO-1 expression as compared to the GM group, suggesting a potential role of HO-1 in the otoprotective effects of fenofibrate.

To confirm the fenofibrate-dependent induction of antioxidant enzymes, we performed immunohistochemistry using the rat cochlear samples. In the controls, the expression of both catalase and SOD-1 was detectable throughout the cochlea, including the spiral ligament, stria vascularis and spiral limbus, while the expression of HO-1 was barely detected (Fig. 4B). GM significantly impaired the expression of catalase and SOD-1, while it increased HO-1 expression. However, pre-treatment with fenofibrate restored the expression of catalase and SOD-1 to a level similar to that of the control, and further increased the expression of HO-1, as compared to that in the GM group. Fenofibrate alone also induced the expression of the antioxidant enzymes to a level similar to, or even higher than that observed in the fenofibrate- and GM-treated group. These results were consistent with the results shown in Fig. 4A, and strongly suggest that fenofibrate prevents GM-induced hair cell death by upregulating the expression of antioxidant enzymes in the rat cochlear explants.

HO-1 inhibitor abolishes the protective effects of fenofibrate on hair cells. Since the activities of catalase and SOD-1 have well been documented for mediating the PPAR-α-dependent protective effects (32,40), we examined whether the strong induction of HO-1 by fenofibrate is essential for hair cell survival. Cochlear explants were treated with SnPPIX, a well-known HO-1 inhibitor, prior to treatment with GM and fenofibrate (SnPPIX + FF + GM). As shown in Fig. 5A, the disruption of sterocilia bundles induced by GM was restored by pre-treatment with fenofibrate, whereas only a moderate recovery was observed in the SnPPIX + FF + GM group. Quantitatively, the number of sensory hair cells in the group pre-treated with fenofibrate was significantly increased in the rat cochlear explants compared to GM group (Fig. 6B). However, the inhibition of HO-1 (SnPPIX + FF + GM) significantly decreased hair cell viability as compared to the pre-treated with fenofibrate and exposed to GM. These results strongly suggest that HO-1 plays an indispensable role in the fenofibrate-mediated protection of sensory hair cells against GM-induced damage.

Fenofibrate protects against GM-induced hair cell death in zebrafish neuromasts. The lateral line of the zebrafish consists of neuromasts aligned along the animal anteroposterior axis (41). Each neuromast contains a group of hair cells that functions to detect water currents via movement of their stereocilia (42) and is used as an alternative for testing ototoxic drugs due to functional and morphological similarities to mammalian hair cells (41). Thus, in the present study, we examined whether the protective effects of fenofibrate are also observed in this model system. Five-day-old zebrafish larvae were pre-treated with 10 μM fenofibrate for 0.5 h and then exposed to 50 μM GM for 1 h. As shown in Fig. 6A, 14 hair cells of the occipital 1 (OC1) and 12 of the posterior 1 (P1) neuromasts were clearly visible in the controls. However, we found that the administration of GM alone significantly decreased the number of neuromast hair cells [OC1, 4.2±2.1 (30±14.17%); P1, 2.9±1.7 (24.2 ±9.29%)], consistent with a previous study in which GM alone was toxic to hair cells in the

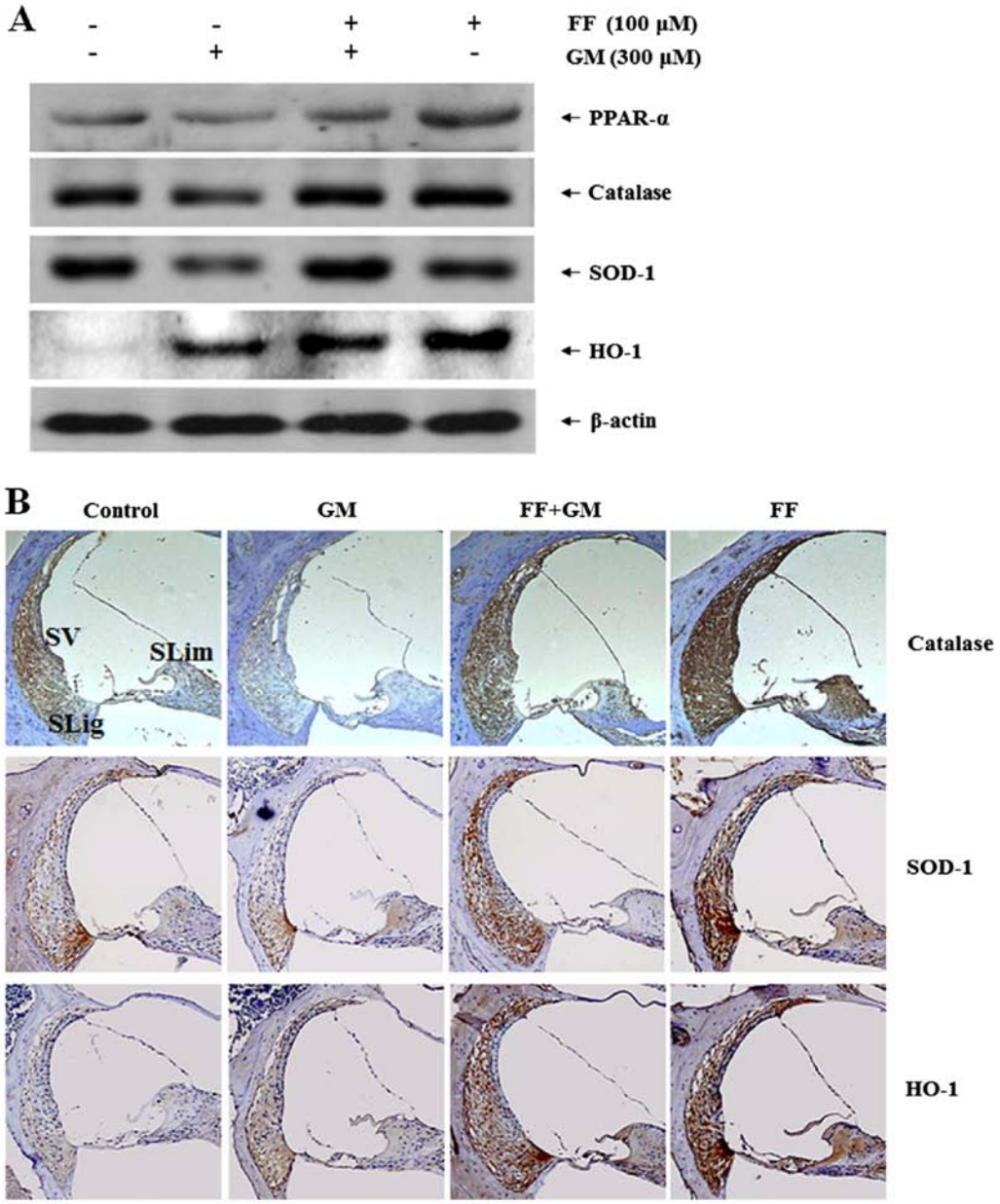

Figure 4. Effect of fenofibrate on the expression of antioxidant enzymes in rat cochlea. (A) Cochlear explants were treated with medium alone, GM (300 μM) for 18 h, fenofibrate (100 μM) pre-treated for 4 h and then co-treated with GM (300 μM) for 18 h, and fenofibrate (100 μM) only for 22 h. The organ of Corti was collected and proteins were extracted. Total cell lysates were separated by 10% SDS-PAGE to detect PPAR-α, catalase, SOD-1 and HO-1 proteins. The protein and mRNA levels of β-actin were determined as controls. (B) The inner ears from SD rat [saline group (control), intraperitoneally injected with 200 mg/kg GM for 4 days (GM), 100 mg/kg fenofibrate for 10 days followed by GM (FF + GM) and fenofibrate alone (FF)] were removed and embedded in paraffin. Next, 4-μm-thick sections were prepared. For immunohistochemistry studies, a commercial kit (LSAB Universal K680) was used to detect the expression levels of catalase, SOD-1 and HO-1 in the cochlear duct regions. GM, gentamicin; FF, fenofibrate; PPAR, peroxisome proliferator-activated receptor; SOD-1, superoxide dismutase-1; HO-1, heme oxygenase-1; SV, stria vascularis; SLig, spiral limbus; SLim, spiral ligament.

lateral line system (43). By contrast, pre-treatment with fenofibrate significantly increased the number of neuromast hair cells [OC1, 10±3.3 (69.3±19.2%); P1, 8.9±2.8 (72.5±17.86%)], indicating at least a partial rescue. Fenofibrate alone did not induce changes to the number of neuromast hair cells. Notably, we found that pre-treatment with SnPPIX and fenofibrate followed by GM (SnPPIX + FF + GM group) significantly decreased the number of neuromast hair cells [OC1, 5.4±1.4 (38.6±10.00%); P1, 4.8±1.2 (40.0±10.00%)], which was similar to the effect observed with GM alone. The survival rate of the neuromast hair cells confirmed that HO-1 activity was required for the protective effects of fenofibrate on sensory hair cells in the zebrafish lateral line (Fig. 6B).

Discussion

GM is one of the most widely used antibiotics. However, its use is restricted due to ototoxicity, including hearing loss and vestibular dysfunction (44,45). The ototoxicity of GM is attributed to the selective loss and/or death of sensory hair cells in the inner ear. Hair cell loss in the cochlea results in acquired permanent hearing loss which, to date, is incurable (46). Therefore, it is

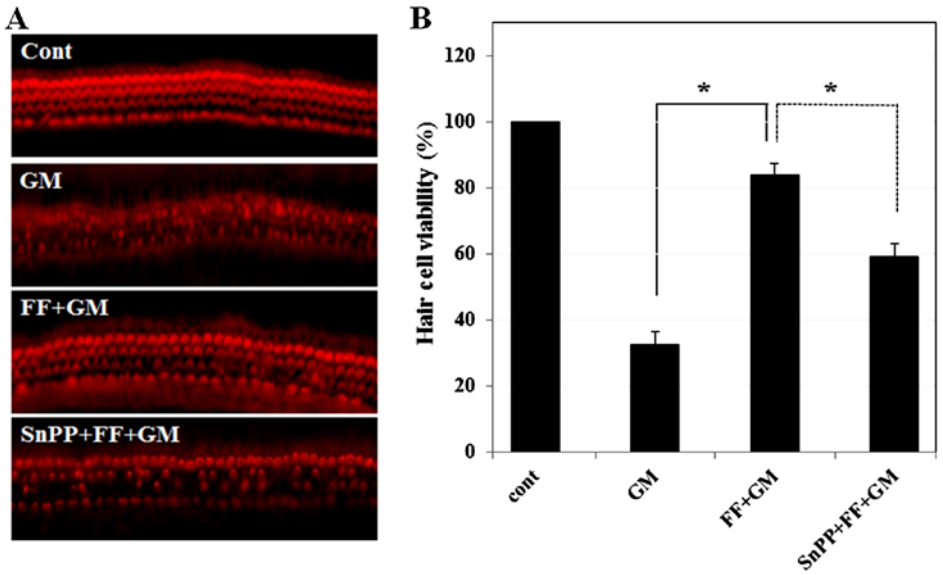

Figure 5. Effect of SnPPIX, an HO-1 inhibitor, on fenofibrate-mediated protection of the sensory hair cells. (A) Sensory hair cells were stained with phalloidin-TRITC and observed under a fluorescence microscope. Cochlear explants were treated with medium alone, GM (300 μM) for 24 h (32.7±3.78, p≤0.00001), pre-treated with fenofibrate (100 μM) for 4 h and then co-treated with GM (300 μM) for 24 h (83.8±3.78, p≤0.00006), and pre-treated with SnPPIX (10 μM) and fenofibrate (100 μM) for 4 h and then further incubated with GM (300 μM) for 24 h (59.3±3.78, p≤0.001). (B) Quantitative analysis of the survival of sensory hair cells. Histogram represents mean hair cell viability. *p<0.001 by one-way ANOVA, compared to GM-treated group or SnPPIX + fenofibrate + GM-treated group. The survival rate (%) of sensory hair cells was calculated using the count method and represented by a bar graph. The number of hair cells was 45±0.0 in control, 14.7±1.7 in GM, 37.7±1.7 in fenofibrate + GM and 26.7±1.7 in SnPPIX + fenofibrate + GM. Note that 10 μM SnPPIX alone did not induce cytotoxicity. GM, gentamicin; FF, fenofibrate; SnPPIX, tin protoporphyrin IX; HO-1, heme oxygenase-1; TRITC, phalloidin-tetramethylrhodamine isothiocyanate.

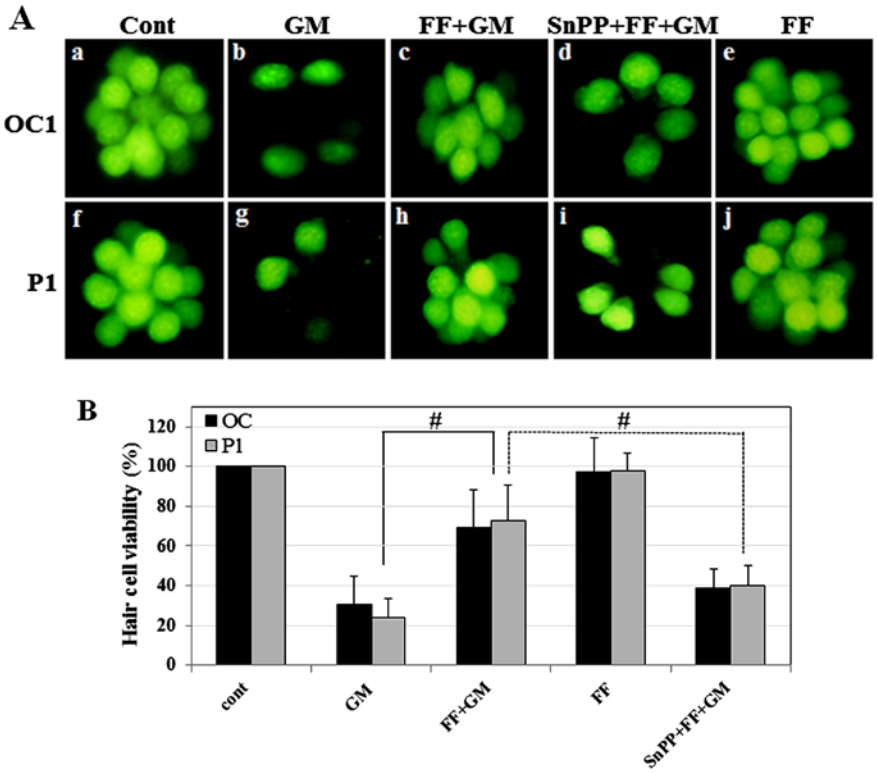

Figure 6. Effect of SnPPIX, a HO-1 inhibitor, on fenofibrate-mediated protection of zebrafish neuromasts. (A) The neuromasts of zebrafish were (50 μM) for 1 h (GM, panels b and g), pre-treated with fenofibrate (10 μM) for 0.5 h and then co-treated with GM (500 μM) for 1 h (FF + GM, panels c and h), pre-treated with SnPPIX (10 μM) and fenofibrate (10 μM) for 0.5 h and then co-treated with GM (50 μM) for 1 h (SnPP + FF + GM, panels d and i), and fenofibrate alone (FF, panels e and j). One of the occipital neuromasts (OC1) is shown in panels a-e, and a posterior neuromast is (P1) shown in panels f-j. (B) Quantitative analysis of neuromast survival. Histogram represents the mean viability of neuromasts. Zebrafish were treated with medium alone (cont), GM (50 μM) for 1 h (OC and P1: 30.7±14.17, p≤ 0.001 and 24.2±9.29, p≤0.0002, respectively), pre-treated with fenofibrate (10 μM) for 0.5 h and then co-treated with GM (500 μM) for 1 h (OC and P1: 69.3±19.2, p≤0.003 and 72.5±17.86, p≤0.0006, respectively), pre-treated with SnPPIX (10 μM) and fenofibrate (10 μM) for 0.5 h and then co-treated with GM (50 μM) for 1 h (OC and P1: 38.6±10.00, p≤0.001 and 40.0±10.00, p≤0.002, respectively), and fenofibrate alone (FF). $^{\#}$p<0.005 by one-way ANOVA, compared to GM or SnPPIX + FF + GM. GM, gentamicin; FF, fenofibrate; SnPPIX, tin protoporphyrin IX; HO-1, heme oxygenase-1.

critical to identify agents that provide protective interventions for aminoglycoside-induced ototoxicity. Several compounds have been introduced as preventive or protective agents against GM-induced ototoxicity (11,47,48). We thus hypothesized that fenofibrate may be a putative preventive therapeutic agent to protect against GM-induced ototoxicity.

GM-induced ototoxicity. Aminoglycoside causes hair cell damage and thus induces prevalent and irreversible ototoxicity (2,3,49). In the present study, we found that GM significantly decreased hair cell numbers in the organ of Corti explants. In addition, ABR experiments revealed that GM induced a significant increase in the hearing threshold in rats. By contrast, fenofibrate prevented hair cell death induced by GM in cochlear explant tissues and significantly attenuated the threshold shifts caused by GM in rats.

Protective effects of fenofibrate are mediated by antioxidant enzymes, including HO-1. Fenofibrate, a PPAR-α activator, belongs to the fibrate drug class. It is mainly used to reduce cholesterol levels in patients at risk of cardiovascular disease (50,51). PPAR-α is one of the three subtypes of PPARs, which have been implicated in several physiological processes, such as the regulation of lipoproteins, lipid metabolism and glucose homeostasis (31). PPARs are ligand-activated transcription factors that belong to the nuclear receptor superfamily (24). Upon activation by their ligands, PPARs regulate gene transcription by binding to PPREs in the promoter regions of target genes as a heterodimer with the retinoid X receptor (52). Previous studies have indicated that PPAR-α activators reduce inflammation by decreasing cytokines, adhesion molecules and nitric oxide synthase 2, and also reduce oxidative stress by increasing antioxidant enzymes in different experimental models (25-30). Furthermore, a PPRE has been identified in the promoter regions of catalase and SOD-1, which are key enzymes involved in reducing ROS production (32). It has been suggested that GM induces the apoptosis of hair cells of the inner ear (53). Since GM-induced cell death is largely mediated by ROS (5-9), several agents that scavenge ROS or block their formation have been proposed to protect the inner ear (10-14). In this study, we found that fenofibrate significantly induced catalase and SOD-1 expression, as shown by western blot analysis and immunohistochemistry, which is consistent with the findings of previous studies (28,40,54). Notably, we found the most prominent increase in response to fenofibrate to be the level of HO-1, whose expression was also induced by GM alone.

HO-1 is a rate-limiting enzyme involved in heme catabolism, which eventually leads to the generation of bilirubin, free iron and carbon monoxide (55). Various oxidative agents induce HO-1 as a stress-responsive protein (20), and several groups have recently reported the versatile functions of HO-1, which protects cells from various oxidative stresses (21-23). A previous study demonstrated the induction of HO-1 expression by PPAR-α and PPAR-γ ligands in cultured vascular cells (56), suggesting that HO-1 may be directly regulated by PPAR. Our previous studies demonstrated a protective role of HO-1 against cisplatin-induced ototoxicity (23,57). In this study, we found that the expression of HO-1 was significantly increased by GM, possibly due to GM-induced oxidative stress (20,57). We also found that the expression of HO-1 was further increased by fenofibrate. Importantly, we demonstrated that SnPPIX, a well-known HO-1 inhibitor, significantly reduced the protective effects of fenofibrate against GM-induced hair cell death in the organ of Corti of adult rats and zebrafish neuromasts, indicating that HO-1 is essential for the protective effects of fenofibrate against GM-induced ototoxicity.

Collectively, our data suggest that the otoprotective role of fenofibrate is mediated by the induction of the expression of antioxidant enzymes, including HO-1. Furthermore, our results strongly suggest that fenofibrate may be used in the development of therapeutic approaches aimed at preventing the extent of acquired hearing loss due to aminoglycoside treatment.

Acknowledgements

This study was supported by National Research Foundation of Korea (NRF) grants funded by the Korea government (nos. 2014M3A9D8034463 and 2011-0030130) and by a grant of the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health and Welfare, Republic of Korea (grant no. HI14C0384).

References

1. Heller J: Effect of some simple manoeuvres on the course of acute renal failure after gentamycin treatment in rats. Int Urol Nephrol 16: 243-251, 1984.
2. Jao RL and Jackson GG: Gentamicin sulfate, new antibiotic against Gram-negative bacilli. Laboratory, pharmacological, and clinical evaluation. JAMA 189: 817-822, 1964.
3. Assimakopoulos D and Patrikakos G: Treatment of Ménière's disease by intratympanic gentamicin application. J Laryngol Otol 117: 10-16, 2003.
4. Chen Y, Huang WG, Zha DJ, Qiu JH, Wang JL, Sha SH and Schacht J: Aspirin attenuates gentamicin ototoxicity: from the laboratory to the clinic. Hear Res 226: 178-182, 2007.
5. Heinrich UR, Helling K, Sifferath M, Brieger J, Li H, Schmidtmann I and Mann WJ: Gentamicin increases nitric oxide production and induces hearing loss in guinea pigs. Laryngoscope 118: 1438-1442, 2008.
6. López-González MA, Lucas M, Delgado F and Diaz P: The production of free oxygen radicals and nitric oxide in the rat cochlea. Neurochem Int 33: 55-59, 1998.
7. Sha SH and Schacht J: Stimulation of free radical formation by aminoglycoside antibiotics. Hear Res 128: 112-118, 1999.
8. Takumida M, Popa R and Anniko M: Free radicals in the guinea pig inner ear following gentamicin exposure. ORL J Otorhinolaryngol Relat Spec 61: 63-70, 1999.
9. Wu WJ, Sha SH and Schacht J: Recent advances in understanding aminoglycoside ototoxicity and its prevention. Audiol Neurootol 7: 171-174, 2002.
10. Jung HW, Chang SO, Kim CS, Rhee CS and Lim DH: Effects of *Ginkgo biloba* extract on the cochlear damage induced by local gentamicin installation in guinea pigs. J Korean Med Sci 13: 525-528, 1998.
11. Sha SH and Schacht J: Antioxidants attenuate gentamicin-induced free radical formation in vitro and ototoxicity in vivo: D-methionine is a potential protectant. Hear Res 142: 34-40, 2000.
12. McFadden SL, Ding D, Salvemini D and Salvi RJ: M40403, a superoxide dismutase mimetic, protects cochlear hair cells from gentamicin, but not cisplatin toxicity. Toxicol Appl Pharmacol 186: 46-54, 2003.
13. Wang AM, Sha SH, Lesniak W and Schacht J: Tanshinone (*Salviae miltiorrhizae* extract) preparations attenuate aminoglycoside-induced free radical formation in vitro and ototoxicity in vivo. Antimicrob Agents Chemother 47: 1836-1841, 2003.
14. Fetoni AR, Sergi B, Ferraresi A, Paludetti G and Troiani D: alpha-Tocopherol protective effects on gentamicin ototoxicity: an experimental study. Int J Audiol 43: 166-171, 2004.

15. Matés JM: Effects of antioxidant enzymes in the molecular control of reactive oxygen species toxicology. Toxicology 153: 83-104, 2000.
16. Chan PH, Yang GY, Chen SF, Carlson E and Epstein CJ: Cold-induced brain edema and infarction are reduced in transgenic mice overexpressing CuZn-superoxide dismutase. Ann Neurol 29: 482-486, 1991.
17. Kinouchi H, Epstein CJ, Mizui T, Carlson E, Chen SF and Chan PH: Attenuation of focal cerebral ischemic injury in transgenic mice overexpressing CuZn superoxide dismutase. Proc Natl Acad Sci USA 88: 11158-11162, 1991.
18. White CW, Avraham KB, Shanley PF and Groner Y: Transgenic mice with expression of elevated levels of copper-zinc superoxide dismutase in the lungs are resistant to pulmonary oxygen toxicity. J Clin Invest 87: 2162-2168, 1991.
19. Mikawa S, Kinouchi H, Kamii H, Gobbel GT, Chen SF, Carlson E, Epstein CJ and Chan PH: Attenuation of acute and chronic damage following traumatic brain injury in copper, zinc-superoxide dismutase transgenic mice. J Neurosurg 85: 885-891, 1996.
20. Alam J and Cook JL: Transcriptional regulation of the heme oxygenase-1 gene via the stress response element pathway. Curr Pharm Des 9: 2499-2511, 2003.
21. He X, Lin GX, Chen MG, Zhang JX and Ma Q: Protection against chromium (VI)-induced oxidative stress and apoptosis by Nrf2. Recruiting Nrf2 into the nucleus and disrupting the nuclear Nrf2/Keap1 association. Toxicol Sci 98: 298-309, 2007.
22. Li HY, Zhong YF, Wu SY and Shi N: NF-E2 related factor 2 activation and heme oxygenase-1 induction by tert-butylhydroquinone protect against deltamethrin-mediated oxidative stress in PC12 cells. Chem Res Toxicol 20: 1242-1251, 2007.
23. Kim SJ, Park C, Han AL, Youn MJ, Lee JH, Kim Y, Kim ES, Kim HJ, Kim JK, Lee HK, *et al*: Ebselen attenuates cisplatin-induced ROS generation through Nrf2 activation in auditory cells. Hear Res 251: 70-82, 2009.
24. Sher T, Yi HF, McBride OW and Gonzalez FJ: cDNA cloning, chromosomal mapping, and functional characterization of the human peroxisome proliferator activated receptor. Biochemistry 32: 5598-5604, 1993.
25. Staels B, Koenig W, Habib A, Merval R, Lebret M, Torra IP, Delerive P, Fadel A, Chinetti G, Fruchart JC, *et al*: Activation of human aortic smooth-muscle cells is inhibited by PPARalpha but not by PPARgamma activators. Nature 393: 790-793, 1998.
26. Inoue I, Goto S, Matsunaga T, Nakajima T, Awata T, Hokari S, Komoda T and Katayama S: The ligands/activators for peroxisome proliferator-activated receptor alpha (PPARalpha) and PPARgamma increase Cu^{2+}, Zn^{2+}-superoxide dismutase and decrease p22phox message expressions in primary endothelial cells. Metabolism 50: 3-11, 2001.
27. Inoue H, Jiang XF, Katayama T, Osada S, Umesono K and Namura S: Brain protection by resveratrol and fenofibrate against stroke requires peroxisome proliferator-activated receptor alpha in mice. Neurosci Lett 352: 203-206, 2003.
28. Toyama T, Nakamura H, Harano Y, Yamauchi N, Morita A, Kirishima T, Minami M, Itoh Y and Okanoue T: PPARalpha ligands activate antioxidant enzymes and suppress hepatic fibrosis in rats. Biochem Biophys Res Commun 324: 697-704, 2004.
29. Lo Verme J, Fu J, Astarita G, La Rana G, Russo R, Calignano A and Piomelli D: The nuclear receptor peroxisome proliferator-activated receptor-alpha mediates the anti-inflammatory actions of palmitoylethanolamide. Mol Pharmacol 67: 15-19, 2005.
30. Bordet R, Ouk T, Petrault O, Gelé P, Gautier S, Laprais M, Deplanque D, Duriez P, Staels B, Fruchart JC, *et al*: PPAR: a new pharmacological target for neuroprotection in stroke and neurodegenerative diseases. Biochem Soc Trans 34: 1341-1346, 2006.
31. Lemberger T, Desvergne B and Wahli W: Peroxisome proliferator-activated receptors: a nuclear receptor signaling pathway in lipid physiology. Annu Rev Cell Dev Biol 12: 335-363, 1996.
32. Girnun GD, Domann FE, Moore SA and Robbins ME: Identification of a functional peroxisome proliferator-activated receptor response element in the rat catalase promoter. Mol Endocrinol 16: 2793-2801, 2002.
33. Hou X, Shen YH, Li C, Wang F, Zhang C, Bu P and Zhang Y: PPARalpha agonist fenofibrate protects the kidney from hypertensive injury in spontaneously hypertensive rats via inhibition of oxidative stress and MAPK activity. Biochem Biophys Res Commun 394: 653-659, 2010.
34. Choung YH, Taura A, Pak K, Choi SJ, Masuda M and Ryan AF: Generation of highly-reactive oxygen species is closely related to hair cell damage in rat organ of Corti treated with gentamicin. Neuroscience 161: 214-226, 2009.
35. Clerici WJ, Hensley K, DiMartino DL and Butterfield DA: Direct detection of ototoxicant-induced reactive oxygen species generation in cochlear explants. Hear Res 98: 116-124, 1996.
36. Hirose K, Hockenbery DM and Rubel EW: Reactive oxygen species in chick hair cells after gentamicin exposure in vitro. Hear Res 104: 1-14, 1997.
37. Sha SH and Schacht J: Formation of reactive oxygen species following bioactivation of gentamicin. Free Radic Biol Med 26: 341-347, 1999.
38. Tanaka Y, Kume S, Araki S, Isshiki K, Chin-Kanasaki M, Sakaguchi M, Sugimoto T, Koya D, Haneda M, Kashiwagi A, *et al*: Fenofibrate, a PPARα agonist, has renoprotective effects in mice by enhancing renal lipolysis. Kidney Int 79: 871-882, 2011.
39. Wang S, Hannafon BN, Zhou J and Ding WQ: Clofibrate induces heme oxygenase 1 expression through a PPARα-independent mechanism in human cancer cells. Cell Physiol Biochem 32: 1255-1264, 2013.
40. Yoo HY, Chang MS and Rho HM: Induction of the rat Cu/Zn superoxide dismutase gene through the peroxisome proliferator-responsive element by arachidonic acid. Gene 234: 87-91, 1999.
41. Chiu LL, Cunningham LL, Raible DW, Rubel EW and Ou HC: Using the zebrafish lateral line to screen for ototoxicity. J Assoc Res Otolaryngol 9: 178-190, 2008.
42. Montgomery J, Carton G, Voigt R, Baker C and Diebel C: Sensory processing of water currents by fishes. Philos Trans R Soc Lond B Biol Sci 355: 1325-1327, 2000.
43. Van Trump WJ, Coombs S, Duncan K and McHenry MJ: Gentamicin is ototoxic to all hair cells in the fish lateral line system. Hear Res 261: 42-50, 2010.
44. Winkel O, Hansen MM, Kaaber K and Rozarth K: A prospective study of gentamicin ototoxicity. Acta Otolaryngol 86: 212-216, 1978.
45. Matz GJ: Aminoglycoside cochlear ototoxicity. Otolaryngol Clin North Am 26: 705-712, 1993.
46. Cotanche DA: Genetic and pharmacological intervention for treatment/prevention of hearing loss. J Commun Disord 41: 421-443, 2008.
47. Garetz SL, Altschuler RA and Schacht J: Attenuation of gentamicin ototoxicity by glutathione in the guinea pig in vivo. Hear Res 77: 81-87, 1994.
48. Ding D, Stracher A and Salvi RJ: Leupeptin protects cochlear and vestibular hair cells from gentamicin ototoxicity. Hear Res 164: 115-126, 2002.
49. Priuska EM and Schacht J: Formation of free radicals by gentamicin and iron and evidence for an iron/gentamicin complex. Biochem Pharmacol 50: 1749-1752, 1995.
50. Forcheron F, Cachefo A, Thevenon S, Pinteur C and Beylot M: Mechanisms of the triglyceride- and cholesterol-lowering effect of fenofibrate in hyperlipidemic type 2 diabetic patients. Diabetes 51: 3486-3491, 2002.
51. Jeong S, Kim M, Han M, Lee H, Ahn J, Kim M, Song YH, Shin C, Nam KH, Kim TW, *et al*: Fenofibrate prevents obesity and hypertriglyceridemia in low-density lipoprotein receptor-null mice. Metabolism 53: 607-613, 2004.
52. Willson TM, Brown PJ, Sternbach DD and Henke BR: The PPARs: from orphan receptors to drug discovery. J Med Chem 43: 527-550, 2000.
53. Forge A and Li L: Apoptotic death of hair cells in mammalian vestibular sensory epithelia. Hear Res 139: 97-115, 2000.
54. Olukman M, Sezer ED, Ulker S, Sözmen EY and Cınar GM: Fenofibrate treatment enhances antioxidant status and attenuates endothelial dysfunction in streptozotocin-induced diabetic rats. Exp Diabetes Res 2010: 828531, 2010.
55. Kikuchi G, Yoshida T and Noguchi M: Heme oxygenase and heme degradation. Biochem Biophys Res Commun 338: 558-567, 2005.
56. Krönke G, Kadl A, Ikonomu E, Blüml S, Fürnkranz A, Sarembock IJ, Bochkov VN, Exner M, Binder BR and Leitinger N: Expression of heme oxygenase-1 in human vascular cells is regulated by peroxisome proliferator-activated receptors. Arterioscler Thromb Vasc Biol 27: 1276-1282, 2007.
57. Kim HJ, So HS, Lee JH, Lee JH, Park C, Park SY, Kim YH, Youn MJ, Kim SJ, Chung SY, *et al*: Heme oxygenase-1 attenuates the cisplatin-induced apoptosis of auditory cells via down-regulation of reactive oxygen species generation. Free Radic Biol Med 40: 1810-1819, 2006.