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Hydrogen protects auditory hair cells from cisplatin-induced free radicals☆

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HIGHLIGHTS

- The use of hydrogen molecule as an antioxidant for inner ear protection is described.
- Cisplatin induced free radical formation and auditory hair cell loss in the cochlea.
- Hydrogen gas increased the number of surviving hair cells after cisplatin damage.
- In hydrogen-treated cochleae, formation of hydroxyl radicals was reduced.

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ABSTRACT

Cisplatin is a widely used chemotherapeutic agent for the treatment of various malignancies. However, its maximum dose is often limited by severe ototoxicity. Cisplatin ototoxicity may require the production of reactive oxygen species (ROS) in the inner ear by activating enzymes specific to the cochlea. Molecular hydrogen was recently established as an antioxidant that selectively reduces ROS, and has been reported to protect the central nervous system, liver, kidney and cochlea from oxidative stress. The purpose of this study was to evaluate the potential of molecular hydrogen to protect cochleae against cisplatin. We cultured mouse cochlear explants in medium containing various concentrations of cisplatin and examined the effects of hydrogen gas dissolved directly into the media. Following 48-h incubation, the presence of intact auditory hair cells was assayed by phalloidin staining. Cisplatin caused hair cell loss in a dose-dependent manner, whereas the addition of hydrogen gas significantly increased the numbers of remaining auditory hair cells. Additionally, hydroxyphenyl fluorescein (HPF) staining of the spiral ganglion showed that formation of hydroxyl radicals was successfully reduced in hydrogen-treated cochleae. These data suggest that molecular hydrogen can protect auditory tissues against cisplatin toxicity, thus providing an additional strategy to protect against drug-induced inner ear damage.

1. Introduction

Cisplatin (cis-diammine-dichloroplatinum (II)/CDDP) is a widely used chemotherapeutic agent in pediatric and adult oncology protocols. Unfortunately, hearing loss is a major dose-limiting side effect that presents as bilateral, irreversible and progressive sensorineural hearing loss, which leads to a decrease in quality of life of cancer patients [12]. Up to 93% of patients receiving cisplatin chemotherapy will experience ear-related symptoms, however, no treatment is currently available for cisplatin-induced ototoxicity [17]. In the inner ear, cisplatin targets the organ of Corti, spiral ganglion neurons (SGNs), the stria vascularis, and spiral ligament [2]. Once cisplatin enters the cell, it induces cell death mainly by oxidative stress and inflammation [18]. Previously, we have shown that this phenomena is caused by apoptotic but not necrotic cell death [15].

Molecular hydrogen (hydrogen gas) has been recently established as a potent antioxidant that selectively reduces the hydroxyl radical, and has been shown to reduce the cerebral infarction volume after ischemia in rats [8,9]. Subsequently, the use of

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hydrogen as a therapeutic medical gas was pursued in diverse models of disease including central nervous system, cardiovascular, gastrointestinal and sensory organs [3–5]. Alleviation of side effects during cancer therapy with hydrogen was also studied using various methods of hydrogen delivery [3,6]. An animal study has demonstrated that consumption of hydrogen-rich water efficiently mitigates cisplatin-induced renal side effects by reducing oxidative stress while retaining the anti-tumor capacity of the drug [7].

This present *ex vivo* study tested the hypothesis that molecular hydrogen protects against cisplatin-induced cochlear impairment. Using a hydrogen gas-saturated culture media, we demonstrated that hydrogen alleviated ROS-induced ototoxicity, suggesting that molecular hydrogen has the potential to serve as an antioxidant for the treatment of cochlear damage. We also evaluated generated hydroxyl radicals by fluorescence emission of 2-[6-(4′-hydroxy)phenoxy-3H-xanthen-3-on-9-yl] benzoate (hydroxyphenyl fluorescein/HPF) in the spiral ganglion and demonstrated that ROS production was successfully reduced in the hydrogen-treated cochlea.

2. Materials and methods

2.1. Animals

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

2.2. Cochlear explant culture

Details for cochlear organotypic cultures were described elsewhere [4]. Briefly, organ of Corti (OC) explants were harvested from postnatal day 2 (P2) ICR mice. The explants were placed on glass-mesh inserts and cultured initially in serum-free modified Eagle's medium supplemented with 3 g/l glucose and 0.3 g/l penicillin G. In total, 20 cochlear explants were used in a single culture, and at least three independent cultures were performed for each condition. Because the hair cells in the apex are resistant to free radicals [14], basal turns of the cochlea were used in this study.

2.3. Cisplatin application

The explants were transferred to medium containing cisplatin (Maruko, Yakult, Japan) at concentrations of 0, 10, 20, or 40 μM with six to nine cochleae incubated at each concentration. The cultures were maintained for 48 h. At the end of the culture period, the samples were fixed for 15 min in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The specimens were then rinsed with PBS and incubated in 1% bovine serum albumin with 0.2% Triton X-100 for 30 min before incubation with primary rabbit polyclonal antibodies against myosin VIIa (1:500; Proteus Bioscience Inc., Ramona, CA). Alexa Fluor 568-labeled goat anti-rabbit IgG (1:200; Invitrogen) was used as the secondary antibody. Specimens were then incubated in Alexa Fluor 488-labeled phalloidin (1:250; Invitrogen) in PBS to visualize the stereocilia. The specimens were examined with a Nikon Eclipse E600 fluorescence microscope (Nikon, Tokyo, Japan) or a Leica TCS-SP2 laser scanning confocal microscope (Leica Microsystems Inc., Wetzlar, Germany), and images were captured with a digital camera (Leica DC330).

2.4. Hydrogen treatment of cultured cells

To evaluate the efficacy of molecular hydrogen for cochlear protection, explants after initial culture were incubated in an airtight container (Chopla Industries, Inazawa, Japan) with reduced-CO_2-dependence media, *i.e.*, MEM and Leibovitz's L-15 media (Invitrogen) mixed in a 1:1 ratio at 37 °C in a humidified 100% air atmosphere with different concentrations of cisplatin, with or without molecular hydrogen. Hydrogen gas was dissolved directly into the media, and a high content of dissolved hydrogen (1.3 ± 0.1 mg/L) was confirmed using a hydrogen electrode (Model M-10B2; Able Corporation, Tokyo, Japan). At the end of the experiments, the explants were fixed and stained with myosin VIIa antibody and phalloidin to evaluate hair-cell survival.

2.5. Cell survival assay

Each cochlea was examined on a Leica TCS-SP2 confocal microscope with a 40× objective, using excitation and emission filters of 488 and 510 nm, respectively. To quantify hair-cell loss in the cochlea after different treatments, inner hair cells (IHCs) and outer hair cells (OHCs) were blindly counted over a 100-μm-long stretch of the auditory epithelia, in two separate randomly selected regions of the basal turn in each cochlear explant (totaling 200 μm). For each treatment, six to nine explants were evaluated.

2.6. Detection of ROS by fluorescent indicators

The spiral ganglion, spiral limbus, and organ of Corti were isolated *en bloc* from P2 ICR mouse cochlear capsule referred to as an 'organotypic' culture. The cultures were then transferred to glass-mesh inserts in an airtight container with or without molecular hydrogen and 40 μM cisplatin. After 100 min, the tissues were treated with 30 μM HPF (Daiichi Pure Chemicals Co., Tokyo, Japan) for 20 min to detect cellular hydroxyl radicals. Fluorescent images were captured with a Leica TCS-SP2 confocal microscope, using a 40× objective (imaged area 280 μm × 280 μm). All images were taken with the same laser intensity, detector gain, and offset values. For each cochlea, consecutive 100 spiral ganglion cells were measured in a randomly selected 7.82×10^4 μm^2 area of the basal turn for HPF fluorescence intensity. This experiment was independently repeated six times.

2.7. Statistical analysis

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

3. Results

3.1. Cisplatin induced dose-dependent hair-cell loss

Initially, we established a dose–response relationship between cisplatin concentration and its toxic effect on hair cells. The addition of cisplatin to cultures for 48 h significantly reduced the hair-cell numbers in both the IHC and OHC regions, with the effect being more severe in the latter (Fig. 1A, C, E, and G). The hair-cell density decreased depending on the concentrations of cisplatin, and few could be detected in the auditory epithelia cultured in 40 μM cisplatin.