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Hydrogen protects auditory hair cells from cisplatin-induced free radicals

Running title: Hydrogen protects cochlea from cisplatin ROS

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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.

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.

Keywords: reactive oxygen species; hydroxyl radical; cochlea; hearing loss; antioxidant; chemotherapy

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 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.

Materials and methods

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.

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.

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).

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 -dependent 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.

Cell survival assay

Each cochlea was examined on a Leica TCS-SP2 confocal microscope with a 40x 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.

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 minutes, 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 40x objective (imaged area 280 μm x 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 $7.82 \times 10^4 \mu\text{m}^2$ area of the basal turn for HPF fluorescence intensity. This experiment was independently repeated six times.

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.

Results

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 μ M cisplatin.

Protective effect of hydrogen supplementation

Subsequently, we assessed the potential of hydrogen to protect against cisplatin-induced ototoxicity by administering 0, 10, 20, or 40 μ M cisplatin to samples cultured in hydrogen-saturated media in an airtight environment. The addition of 10, 20 or 40 μ M cisplatin destroyed $1.9 \pm 15.1\%$, $13.0 \pm 14.0\%$ or $62.0 \pm 23.0\%$ of the IH cells and $63.6 \pm 15.1\%$, $89.1 \pm 4.1\%$ or $90.0 \pm 11.1\%$ of the OH cells, respectively (mean \pm SEM). When hydrogen was added, the cell loss rates were $1.7 \pm 21.9\%$, $12.7 \pm 23.5\%$ or $39.0 \pm 11.2\%$ for IHC and $46.1 \pm 3.4\%$, $68.4 \pm 11.4\%$ or $87.2 \pm 14.7\%$ for OHC,

respectively. The addition of hydrogen increased both IHC and OHC survival, with a substantial number of inner hair cells surviving even at the highest cisplatin dose (40 μ M; Fig. 1I). Two-way factorial ANOVA showed that hydrogen had a significant effect on the numbers of surviving IHCs ($P = 0.0008$) and OHCs ($P = 0.0005$) in cisplatin-damaged cochleae. However, post-hoc analysis could not identify any individual factor responsible for these statistically significant changes. No significant interaction between the two factors (concentration of cisplatin and presence of hydrogen) was found.

ROS production in the cochlea was attenuated by molecular hydrogen

To investigate the mechanism by which hydrogen alleviated cochlear damage, we further measured ROS production in the cultured auditory epithelia (Fig. 2). HPF is a novel reagent that was developed to directly detect certain highly reactive oxygen species (hROS) [13]. Although HPF itself shows little fluorescence, it selectively and dose-dependently reacts with hROS, such as hydroxyl radicals and peroxynitrite, to generate the strongly fluorescent compound fluorescein. In cochlear organotypic cultures treated with 40 μ M cisplatin for 100 min and in the absence of molecular hydrogen, HPF signals increased, indicating that cisplatin induced the production of hROS. In the spiral ganglion, the intensity of the HPF fluorescence after treatment with 40 μ M cisplatin was 1.19-fold greater than that in the absence of cisplatin (Fig. 2A, C). In contrast, with hydrogen, HPF fluorescence intensity increased by 1.07-fold, which indicates that hROS production was suppressed after hydrogen application. Two-way ANOVA showed that hydrogen had a significant effect on the formation of hydroxyl radicals ($P = 0.036$).

Discussion

The exact mechanism for cisplatin ototoxicity still remains unclear. However, it appears that cisplatin causes apoptosis by binding DNA, activating the inflammatory cascade as well as generating oxidative stress in the cell [17]. Cisplatin in the cochlea causes the formation of reactive oxygen radicals, especially free radicals, such as superoxide anions. Cisplatin ototoxicity is also believed to be related to malfunction of the antioxidant system. Derangement of the antioxidant defense system causes an increase in lipid/DNA peroxidation, and therefore leads to apoptosis of hair cells, support cells, the stria vascularis, and auditory nerves [16].

We have previously reported the effects of hydrogen gas on cochlear and vestibular hair cells against ROS toxicity with antimycin A, which inhibits the mitochondrial electron transport complex III and produces ROS [4] [15]. In this study, we applied this same method to cisplatin-damaged cochlea. Our cochlear culture system, along with chemiluminescence detection, was highly useful in screening for antioxidant drugs, because ROS was directly produced in the cochlear tissue and the antioxidative effect can easily be measured by counting the number of surviving cells. In this study, quantitative assessment of hair cell loss caused by cisplatin showed a dose-dependent effect, and treating the cultures with molecular hydrogen significantly influenced the dose response for hair cell loss, indicating that molecular hydrogen has a protective effect on the hair cells against cisplatin toxicity. Obviously the situation *ex vivo* differs in many respects from that *in vivo*, with direct access of the drug to the apical surface of the hair cell or tissues like stria vascularis and spiral ligament were excised in the dissection process. Nonetheless this culture system is at least one in which morphological responses to cisplatin can be rapidly observed under strictly controlled conditions.

We also investigated the mechanisms by which molecular hydrogen protected hair

cells from damage in the cochlea. The direct measurement of ROS in the cochlear tissue was made possible when HPF was used. Our results demonstrated that molecular hydrogen reduced cellular ROS in the auditory tissue. Nakashima-Kamimura showed that consuming hydrogen water *ad libitum* alleviates ROS production in the kidney, thus preventing acute renal injury, reduced body weight loss and mortality after systemic cisplatin administration [7].

Hydrogen is one of the most abundant and well-known molecules. Inhalation of molecular hydrogen gas has been used to prevent decompression sickness in divers, has shown a good safety profile, and has been approved by the US Food and Drug Administration for the treatment of several different diseases [3, 8]. Molecular hydrogen is a mild but potent antioxidant with certain unique chemical properties; being a gas allows it to be permeable to cell membranes, and it can therefore directly target cellular organelles. This feature is especially favorable in inner ear medicine, because many therapeutic compounds are blocked by the blood–cochlear barrier and cannot reach the organ of Corti and cochlear hair cells [1]. There are several ways to intake or consume hydrogen, including inhaling hydrogen gas, drinking hydrogen-dissolved water, taking a hydrogen bath, injecting hydrogen-dissolved saline, and increasing the production of intestinal hydrogen by bacteria [9, 10]. Lin *et al.* [5] utilized hydrogen-dissolved drinking water to treat noise-induced hearing loss. Another possible approach is injecting hydrogen-dissolved saline to the middle ear, which allows the dissolved hydrogen to immediately penetrate to the cochlea.

Previous studies have aimed at evaluating various compounds such as antioxidants, anti-inflammatories, caspase inhibitors and calcium channel blockers against the toxicity caused by cisplatin in the inner ear with variable degrees of protection [11, 17]. The

greatest level of otoprotection seems to arise from antioxidant treatments, however, the ideal protective agent has not yet been found [17]. Therefore, many researchers have suggested a combination of antioxidants to pursue maximum effect and safety [11]. Our present study proposes hydrogen as an additional approach to protect against drug-induced inner ear damage.

Conclusions

This study demonstrated that molecular hydrogen protected cochlear hair cells against cisplatin-induced toxicity. Hydrogen decreased oxidative stress by scavenging ROS and protected cochlear tissue against cisplatin-induced oxidative stress. These results have prompted us to perform *in vivo* studies to determine whether treatment with molecular hydrogen might exert a beneficial effect on cisplatin-damaged cochleae and promote hearing recovery.

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and Health.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

Figure

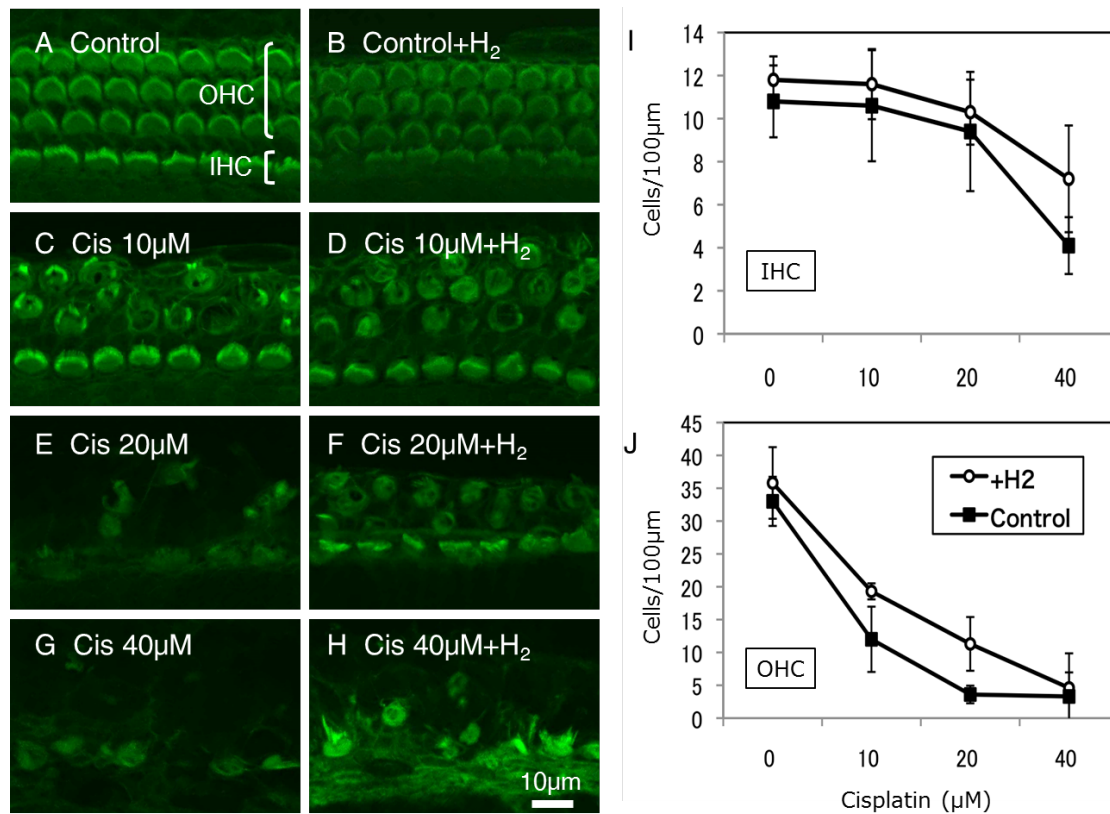


Figure 1: Effect of molecular hydrogen (H₂) on the survival of cochlear hair cells. (A–H) Photomicrographs of phalloidin-labeled cochlear cultures treated with 0 (A and B), 10 (C and D), 20 (E and F), and 40 μM (G and H) cisplatin (Cis), with (B, D, F and H) or without (A, C, E and G) hydrogen gas. IHCs, inner hair cells; OHCs, outer hair cells. Note that cisplatin effects are greater on inner OHCs than IHCs. Bar, 10 μm. (I–H) Hair-cell counts in hydrogen-treated cultures. After a 48-h culture with cisplatin, IHCs (I) and OHCs (J) were counted. Black box symbols represent the counts from control cultures without hydrogen, and white circles represent those from cultures with molecular hydrogen. Two-way factorial ANOVA showed molecular hydrogen significantly attenuated the loss of IHCs ($P = 0.0008$) and OHCs ($P = 0.0005$) in cisplatin-damaged cochleae. Bars represent standard errors.

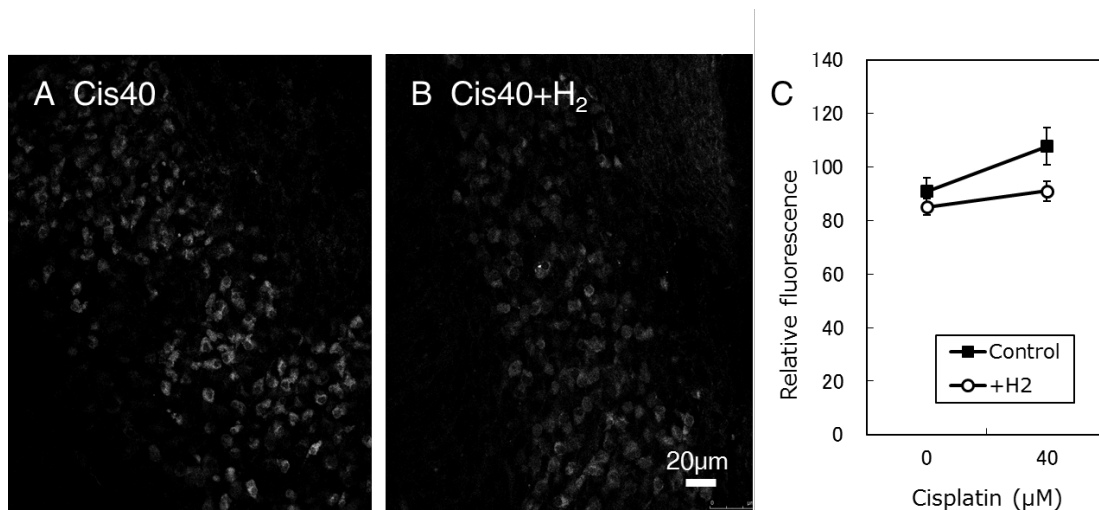


Figure 2: Molecular hydrogen (H₂) reduced reactive oxygen species (ROS) formation.

(A, B) Photomicrographs of HPF in spiral ganglion cultures treated with 40 μM cisplatin for 100 min with (B) or without (A) hydrogen gas (H₂). Bar, 20 μm. (C) Relative HPF fluorescence intensity in the spiral ganglion cells. Black box symbols represent the counts from control cultures without molecular hydrogen, and white circle symbols represent those from cultures with molecular hydrogen ($n = 6$, 100 cells were measured for each culture). The relative HPF fluorescence intensity in the spiral ganglion increased in the presence of cisplatin but declined significantly with the addition of hydrogen gas ($P = 0.036$). The data shown are the relative intensities of HPF staining \pm SE ($n = 6$ cochleae). Bars represent standard errors.

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