ORIGINAL ARTICLE

Hydrogen protects vestibular hair cells from free radicals

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Abstract

Conclusion: Hydrogen gas effectively protected against the morphological and functional vestibular hair cell damage by reactive oxygen species (ROS). Objective: ROS are generally produced by oxidative stress. In the inner ear, ROS levels increase as a result of noise trauma and ototoxic drugs and induce damage. It is thus important to control ROS levels in the inner ear. The protective effects of hydrogen gas in cochlear hair cells have been reported previously. Methods: This study examined the effects of hydrogen gas on mouse vestibular hair cell damage by ROS using antimycin A. Results: In the group *exposed to hydrogen gas, vestibular hair cells were morphologically well preserved and their mechano-electrical transduction activities were relatively well maintained when compared with controls. Hydroxyphenyl fluorescein (HPF) fluorescence in vestibular tissue was also reduced by hydrogen gas.

Keywords: Reactive oxygen species, vestibular hair cells, mechano-electrical transduction

Introduction

Numerous patients suffer from balance disorders and the number increases with age. Vestibular hair cells also degenerate with age [1] and are damaged by oxidative stress [2]. Oxidative stress is one of the most important factors related to disorders of the inner ear [3–5]. In the inner ear, levels of reactive oxygen species (ROS) increase as a result of noise trauma and ototoxic drugs [6–10] and induce damage. It is thus important to control ROS levels in the inner ear.

Hydrogen molecules have recently been reported to act as an antioxidant that selectively reduces hydroxyl radicals, and have been shown to decrease cerebral infarction volume after ischemia [11]. Hydrogen gas also shows protective effects in hepatic injury [12], myocardial infarction [13], and glucose metabolism in patients with type 2 diabetes [14]. In the cochlea, Kikkawa et al. reported that hydrogen gas alleviated ROS-induced ototoxicity [15]. We thus applied their method to vestibular hair cells and investigated hair cell function. The function of hair cells was monitored by accumulation of FM1-43, which is known to reflect the activity of mechano-electrical transducer (MET) channels [16]. We also evaluated the generated hydroxyl radicals by fluorescence emission of 2-[6-(40-hydroxy)-phenoxy-3H-xanthen-3-on-9-yl] benzoate (HPF) to examine the protective mechanisms.

Material and methods

Animals

ICR mice at 2 postnatal days (P2) were used in this study and were purchased from Shimizu Experimental Animals (Hamamatsu, Japan). The Animal Research Committee of the Kyoto University Graduate School of Medicine approved all experimental protocols.

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Vestibular maculae explant culture

P2 ICR mice were deeply anesthetized with diethyl ether and decapitated. The temporal bones were dissected and the vestibular maculae were freed from the surrounding tissue and placed in 0.01 M phosphate-buffered saline (PBS; pH 7.4). After removing the otoconia, samples were placed on 12 mm collagen-coated cover glasses (4912-010, Iwaki, Asahi Glass Co. Ltd, Tokyo, Japan), followed by culture in serum-free modified Eagle’s medium (MEM; Invitrogen, Eugene, OR, USA) supplemented with 3 g/l glucose (Wako Pure Chemicals, Osaka, Japan) and 0.3 g/l penicillin G (Wako), for 24 h at 37°C in humidified (95%) air:5% CO2.

Antimycin A application and hydrogen treatment

Explants were incubated in medium containing antimycin A (Sigma-Aldrich, St Louis, MO, USA) at a final concentration of 1 µg/ml. Cultures were maintained for 24 h. At the same time, explants were cultured initially in an airtight box (Chopla Industries, Inazawa, Japan) with reduced CO2-dependence media, MEM, and Leivovitz’s L-15 medium (Invitrogen, CA, USA) mixed in a 1:1 ratio [14], supplemented with 3 g/l glucose and 0.3 g/l penicillin G, at 37°C in humidified (100%) atmospheric air. After 24 h, the medium was changed to one containing antimycin A at a concentration of 1 µg/ml, with or without hydrogen gas for another 24 h. 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). The pH of the culture media without hydrogen gas was 7.18 ± 0.02, and that of culture media with hydrogen gas was 7.52 ± 0.02. The prepared media were used for culture within 30 min.

Immunohistological analysis

At the end of the culture period, samples were fixed for 15 min in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), and then provided for immunostaining for myosin VIIa to evaluate the number of surviving hair cells. Specimens were incubated with primary rabbit polyclonal antibodies against myosin VIIa (1:500; Proteus Bioscience Inc., Ramona, CA, USA). Alexa-Fluor 568 goat anti-rabbit IgG (1:100; Invitrogen, CA, USA) was used as the secondary antibody. Specimens were then incubated in Alexa-Fluor 488-conjugated phalloidin (1:100; Invitrogen) to label F-actin. Specimens were examined with a Leica TCS-SP2 laser-scanning confocal microscope (Leica Microsystems Inc., Wetzlar, Germany). To quantify hair cell loss after treatments, hair cells were counted in more than three regions of vestibular epithelia.

Counting of remaining hair bundles

Remaining hair bundles were measured using a 10 × 40 eyepiece reticle. Each square of the reticle was 100 µm on each side. Remaining hair bundles were counted in each of three randomly selected fields containing both striolar and extrastriolar regions, and the values obtained were averaged. At least 10 vestibules were examined for each set of conditions.

FM1-43 accumulation of hair cells

A lipophilic dye, FM1-43 FX (Invitrogen), which has been shown to enter hair cells through transducer channels [16], was used to detect hair cells with active transducer channels by accumulation of FM1-43FX (5 µM FM1-43 in PBS, prepared from 10 mM stock solution in DMSO). The mechanical-electrical transduction activity of hair cells was quantified based on dye accumulation by applying mechanical stimulation for 10 s [16]. Subsequently, explants were washed three times with PBS for 10 min each, followed by fixing with 4% PFA. FM1-43 accumulation within the cells was observed under a fluorescence microscope.

HPF analysis

At the end of the experiments, explants were treated with 30 mM 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. All images were taken under the same laser intensity, detector gain, and offset values.

Statistical analysis

Quantitative differences were evaluated by two-way factorial analysis of variance (ANOVA). Significance was evaluated at a level of p < 0.05. Data are presented as means and standard errors, along with the number of explants under each condition.
Results

Protective effects of hydrogen gas on morphology

The effects of hydrogen gas on the vestibule are illustrated in Figure 1. In the absence of hydrogen gas, most hair bundles were eliminated and the number of myosin VIIa-positive cells was small (Figure 1A and C). However, in explants treated simultaneously with hydrogen gas, hair bundles tended to be preserved and numerous myosin VIIa-positive cells were observed (Figure 1B and D). The morphology of hair bundles without hydrogen gas was severely damaged (Figure 2A). Some of them fused with each stereocilia or were fanned out. In contrast, the morphology of each hair bundle was preserved intact in the presence of hydrogen gas (Figure 2B). Scale bar: 10 μm.

Figure 1. Effects of hydrogen gas in the vestibule. In the absence of hydrogen gas, most hair bundles were eliminated and the number of myosin VIIa-positive cells was small (A and C). However, in explants treated simultaneously with hydrogen gas, hair bundles were preserved and numerous myosin VIIa-positive cells were observed (B and D). Scale bar: 50 μm.

Figure 2. Morphology of hair bundles. The hair bundle without hydrogen gas was severely damaged (A). Some of them fused with each stereocilia or were fanned out. In contrast, the morphology of each hair bundle was preserved intact in the presence of hydrogen gas (B). Scale bar: 10 μm.
severely disrupted (Figure 2A). Some bundles showed fused or fanned out stereocilia. In contrast, the morphology of hair bundles was preserved in the presence of hydrogen gas (Figure 2B). The number of remaining hair bundles in the groups treated with hydrogen gas was significantly larger when compared with the group treated with antimycin A alone ($p < 0.05; n = 19$) (Figure 3).

Protective effects of hydrogen gas on function

The effects of hydrogen gas on vestibular hair cell function are illustrated in Figure 4.

In the absence of hydrogen gas, some hair cells showed FM1-43 accumulation (Figure 4A). However, in explants treated simultaneously with hydrogen gas, numerous hair cells showed FM1-43 accumulation (Figure 4B). The number of FM1-43-positive hair cells in the group treated with hydrogen gas was significantly larger when compared with the group treated with antimycin A alone ($p < 0.0001; n = 12$) (Figure 4C).

**Figure 3.** Number of remaining hair bundles. In the groups treated with hydrogen gas, the number of remaining hair bundles was significantly larger when compared with the group treated with antimycin A alone ($p < 0.05; n = 19$).

**Figure 4.** Effects of hydrogen gas on the function of vestibular hair cells. In the absence of hydrogen gas, few hair cells showed FM1-43 accumulation (A). However, in explants treated simultaneously with hydrogen gas, numerous hair cells showed FM1-43 accumulation. Scale bar: 20 µm (B). The number of FM1-43-positive hair cells in the group treated with hydrogen gas was significantly larger when compared with the group treated with antimycin A alone ($p < 0.0001; n = 12$); bar: SE (C).
accumulation (Figure 4B). The number of FM1-43-positive hair cells in the groups treated with hydrogen gas was significantly larger when compared with the group treated with antimycin A alone \((p < 0.0001; n = 12)\) (Figure 4C).

**ROS reduction by hydrogen gas**

To investigate the mechanisms of protection, we measured ROS production in explants (Figure 5). HPF is a reagent that was developed to directly detect certain ROS \([17]\). There were numerous HPF-positive cells after treatment with antimycin A (Figure 5A). In contrast, there were few HPF-positive cells in the group treated with hydrogen gas (Figure 5B). The number of HPF-positive hair cells in the group treated with hydrogen gas was significantly smaller when compared with the group treated with antimycin A alone \((p < 0.0001; n = 10)\) (Figure 5C).

**Discussion**

Kikkawa et al. \([15]\) previously reported the effects of hydrogen gas on cochlear hair cells against ROS toxicity with antimycin A. In this study, we used the same method and applied it to vestibular hair cells. Initially, we used three different antimycin A concentrations, and we found that 10 \(\mu\)g/ml resulted in severe damage and 0.01 \(\mu\)g/ml resulted in insufficient damage (data not shown). Our results showed that hydrogen gas has protective effects on vestibular hair cells against ROS toxicity by reducing the production of ROS.

![Figure 5. HPF analysis. There were numerous HPF-positive cells after treatment with antimycin A in the absence of hydrogen gas (A). In contrast, there were a few HPF-positive cells in the group treated with hydrogen gas (B). The number of HPF-positive hair cells in the group treated with hydrogen gas was significantly smaller when compared with the group treated with antimycin A alone (bar: SE; \(*p < 0.0001; n = 10\) (C).]
There are two hypotheses regarding the mechanisms by which ROS induce cell damage; ROS may induce activation of caspase-3, which causes apoptosis [18], or ROS may induce increases in calcium concentration, thus leading to necrosis [19]. In our experiment, activation of caspase-3 was observed in the group treated with antimycin A alone (data not shown). Thus, it is possible that hydrogen gas reduces the production of ROS and prevents the activation of caspase-3, thus preventing apoptosis.

Ohsawa et al. [11] demonstrated that hydrogen gas has some unique properties. Hydrogen gas can permeate the blood–brain barrier, which is very important in the inner ear, as it is difficult for medicines to reach hair cells because of this barrier [20,21]. Hydrogen gas also has specific activity against detrimental ROS, such as the hydroxyl radical and peroxynitrite, while the metabolic oxidation-reduction reaction and other less potent ROS are not affected. Therefore, hydrogen gas may be a promising treatment for balance disorders. However, further basic and clinical investigations are required.

Conclusion

In conclusion, we showed that hydrogen gas markedly decreases oxidative stress by scavenging ROS, thus protecting vestibular hair cells against oxidative stress. These results have prompted us to perform in vivo studies to determine whether treatment with hydrogen gas exerts beneficial effects on damaged vestibules to ameliorate balance disorders.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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