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Post-exposure administration of edaravone attenuates noise-induced hearing loss

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Abstract

We investigated the effects of the antioxidant edaravone against acoustic trauma in guinea pigs. Edaravone (1.722×10^{-2} M) was infused into the right ear by an osmotic pump, and the left ear was untreated for control. Animals received edaravone 9 h before (−9 h group, $n=7$) and 9 h (+9 h group, $n=8$), 21 h (+21 h group, $n=7$) and 33 h (+33 h group, $n=4$) after 3-h exposure to 130-dB noise. Seven days after noise exposure, we examined the shift in auditory brainstem response thresholds and histopathologic characteristics of the sensory epithelia. The smallest shift in auditory brainstem response threshold and smallest proportion of missing outer hair cells were observed in the +9 h group. This result was supported by immunohistochemical analysis of 4-hydroxy-2-nonenal. Our data suggest that edaravone may be clinically effective in the treatment of acoustic trauma, especially if given within 21 h of noise exposure.

Keywords: Edaravone; Cochlear protection; Antioxidant; Reactive oxygen species; Acoustic trauma; 4-HNE(4-hydroxy-2-nonenal)

1. Introduction

Noise exposure leads to increased levels of reactive oxygen species in the cochlea (Yamane et al., 1995), and noise-induced hearing loss can be reduced by treatment with antioxidants (Yamasoba et al., 1999; Kopke et al., 2000; Ohinata et al., 2000, 2003; McFadden et al., 2001; Lynch et al., 2004). However, these drugs have not been used in clinical settings.

Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-1) is the first free-radical scavenger used in clinical practice in Japan, where it is used to treat acute cerebral infarction (Yamamoto et al., 1997). There are some studies showing an effect of edaravone on the inner ear (Horiike et al., 2003, 2004; Maetani et al., 2003; Takemoto et al., 2004). Takemoto et al. (2004) reported that pre-exposure perilymphatic application of edaravone reduced noise-induced hearing loss in guinea pigs. Thus, in the present study, we administered edaravone to guinea pigs before and after noise exposure, and investigated the optimal timing for administration of edaravone to protect the cochlea. In addition, at various time points after noise exposure we measured levels of 4-hydroxy-2-nonenal (4-HNE), which is a phospholipid peroxidation product generated by the reaction of free radicals with the plasma membrane.

2. Materials and methods

2.1. Animals

We used 34 male Hartley guinea pigs (300–450 g each; Chiyoda, Tokyo, Japan) with normal Preyer's reflexes and normal tympanic membranes. Twenty-six animals were divided into four treatment groups as described below, and 8 animals were used for immunohistochemical analysis of 4-HNE, also described below. This experiment was reviewed by the Committee for the Ethics of Animal Experiments in Yamaguchi University School of Medicine and carried out according to the Guideline for Animal Experiments of Yamaguchi University School of Medicine and The Law (No. 105) and Notification No. 6 of the Japanese Government.

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2.2. Pump implantation

An osmotic pump (Model 2002, Alza Co., Palo Alto, CA, USA) filled with saline was implanted in the right ear of 26 guinea pigs, and the left ear was kept intact as a control. The pigs were anesthetized with a mixture of ketamine (16 mg/kg, i.p.) and xylazine (16 mg/kg, i.p.). After hypodermic injection of 1.5 ml 1% lidocaine, the temporal bone was exposed via postauricular incision. The mastoid bulla was opened with a 4-mm diamond burr to allow visualization of the round window. A tiny hole was made with a needle at a distance of 1 mm from the round window. The tip of the catheter was inserted into the hole, and saline was infused into the perilymphatic space of the cochlea. The polyethylene catheter was fixed to the mastoid bulla with dental cement (GC Fuji I, GC Co., Tokyo, Japan). The skin incision was closed and treated with antibiotic. The flow rate of the pump was 0.5 l/h. The osmotic pump was connected to a 10-cm polyethylene catheter (inner diameter=0.28 mm, outer diameter=0.61 mm; Becton Dickinson, Franklin Lakes, NJ, USA) and a 1-mm Teflon catheter (inner diameter=0.18 mm, outer diameter=0.3 mm; Unique Medical, Tokyo, Japan). The pump and catheter were filled with saline.

2.3. Pump exchange

The implanted osmotic pump was replaced by another pump filled with 1.722×10^{-2} M edaravone (Mitsubishi Pharma Co., Osaka, Japan), the concentration used in a previous in vivo study (Yamamoto et al., 1997). Edaravone was dissolved in 1 mol/l NaOH and water just prior to use, and the pH was adjusted to 7 with 1 mol/l HCl. The pumps were replaced under xylazine and ketamine general anesthesia. After hypodermic injection of 1.5 ml 1% lidocaine, an incision was made on each animal's back, and the saline-filled osmotic pump was replaced by an edaravone-filled pump. After the incision was closed, antibiotic ointment was applied to the incision site. The catheter was designed to begin administration of edaravone to the cochlear perilymph 12 h after pump exchange. In one group of animals, pumps were changed 21 h before noise exposure, and edaravone administration was started 9 h before noise exposure (−9 h group, $n=7$). In another group, pumps were changed immediately before noise exposure, and edaravone administration was started 9 h after noise exposure (+9 h group, $n=8$). In a third group and a fourth group, pumps were changed 9 and 21 h, respectively, after noise exposure, and edaravone administration was started 21 and 33 h, respectively, after noise exposure (+21 h group, $n=7$, and +33 h group, $n=4$, respectively).

2.4. Noise exposure

Guinea pigs under pentobarbital anesthesia (33 mg/kg, i.p.) were exposed to intense (130 dB sound pressure level) band noise centered at 4 kHz for 3 h. The noise we used was designed to cause permanent threshold shifts and to damage cochlear hair cells at the basal end of the second turn (Yamasoba et al., 1999). Each animal was immobilized, and a speaker was centered over the animal's head at a distance of 15 cm. The sound intensity was monitored with a sound-level meter (NA-60, Rion, Tokyo, Japan) positioned near the external auditory canal.

2.5. Auditory brainstem responses

The auditory brainstem response threshold was examined in guinea pigs under xylazine (16 mg/kg, i.p.) and ketamine (16 mg/kg, i.p.) anesthesia 3 days after pump implantation and 7 days after noise exposure. Responses were recorded between subcutaneous stainless steel electrodes located at the vertex (positive) and antinion (negative); the lower back served as the ground. The sound stimuli consisted of 2-, 4-and 8-kHz tone bursts (rise–fall time 2 ms, duration 4 ms). Stimuli were presented through a 10-cm-long tube that connected an earphone to the external auditory canal. The stimulus intensity was evaluated with a NA-60 sound-level meter adjacent to the tip of the tube. Responses to 500 stimuli were recorded with a signal processor (Synax 1100, NEC Co., Tokyo, Japan). The auditory brainstem response threshold was defined as the lowest stimulus intensity that produced a reliable waveform of 3–5 peaks.

2.6. Histopathology

One week after noise exposure, auditory brainstem response thresholds were recorded, and the 26 guinea pigs were killed with an overdose of pentobarbital. Both temporal bones of each animal were removed. The cochlea was opened at the apex, base and oval window and perfused with fixative (4% paraformaldehyde in 0.01 M phosphate-buffered saline (PBS), pH 7.3) gently for 12 h. The cochlea was rinsed in PBS, and the organ of Corti in the second turn of the cochlea was removed. The specimen was permeabilized with 0.3% Triton X-100 (Katayama Chemical Inc., Osaka, Japan) for 10 min and subsequently incubated with fluorescein isothiocyanate-conjugated phalloidin (1:50 dilution; Sigma, St. Louis, MO, USA) at room temperature for 1 h. The specimen was rinsed in PBS and then mounted using a SlowFade Light Antifade Kit (Molecular Probes, Eugene, OR, USA). The surface structure of each specimen was observed under a fluorescence microscope (Zeiss, Jena, Germany). The missing inner hair cells and outer hair cells in the second turn of the cochlea were counted, and the percentage of missing outer hair cells was recorded.

2.7. Immunohistochemistry for 4-HNE

We examined 4-HNE production in animals that did not receive edaravone. Animals were exposed to the same experimental noise conditions and killed at three different time points (immediately, 9, and 21 h) after noise exposure ($n=2$ each group). Two animals that were unexposed served as controls ($n=2$). The temporal bones were removed under deep pentobarbital anesthesia and transferred to 4% paraformaldehyde as described above and kept in fixative overnight. The tissue was decalcified in 5% ethylene diamine tetraacetic acid (pH 7.2) for approximately 14 days. The lateral walls of the cochleae were removed with a micromanipulator.

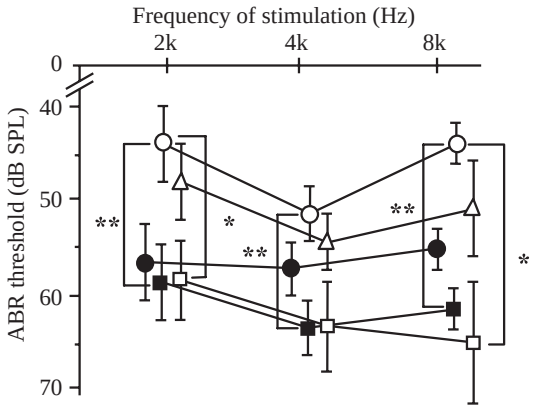

Fig. 1. Auditory brainstem response thresholds of edaravone-treated ears in the −9h group (closed circles), +9 h group (open circles), +21 h group (open triangles) and +33 h group (open squares) and control ears in all groups (closed squares) 1 week after noise exposure. The smallest auditory brainstem response threshold shifts were seen in the treated ears in the +9 h group. Error bar±1 S.E.M., $**P<0.01$, $*P<0.05$.

Specimens were rinsed in PBS, incubated in methanol for 20 min at −20 °C, and then hydrated in PBS containing 0.1% Triton X-100 for 10 min. Specimens were soaked in PBS/2% dried milk for 2 h at 4 °C and then incubated for 12 h at 4 °C in a 1:100 dilution of anti-4-HNE mouse monoclonal antibody (OXIS International, Inc., Portland, OR, USA). The specimens were rinsed in PBS and incubated for 8 h at 4 °C in a 1:100 dilution of Alexa Fluor® 568-conjugated goat anti-mouse IgG (Molecular Probes). They were then rinsed in PBS and embedded in a semi-water-soluble resin (Immuno-Bed®, Polysciences, Inc., Washington, DC, USA) and cut into 2-μm-thick sections. Sections were counterstained with 4′ 6-diamino-2-phenylindole (DAPI) (Vectashield®, Vector Laboratories, Inc., Burlingame, CA, USA). Immunolabeling was visualized under brightfield illumination by means of a fluorescence microscope with a 20× objective.

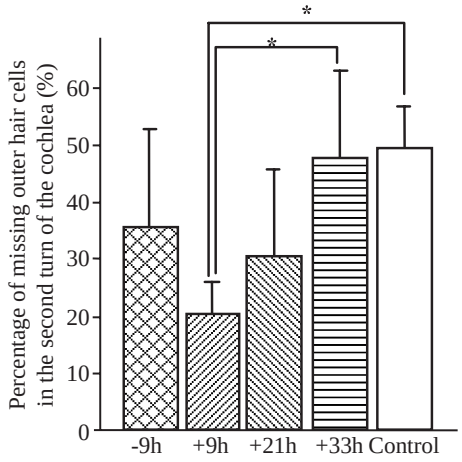

Fig. 2. Percentage of missing outer hair cells in the second turn of the cochlea 1 week after noise exposure. The number of missing outer hair cells was lower in the edaravone-treated ears than in the control ears in the −9, +9 and +21 h groups. In the +33 h group, there was no significant difference between edaravone-treated and control ears. Error bar±1 S.E.M., $*P<0.05$.

2.8. Statistical analysis

The differences in post-exposure auditory brainstem response thresholds and percentages of missing outer hair cells among the four groups and between treated and untreated ears were analyzed by Mann–Whitney *U*-test with StatView version 4.5J for Macintosh (Abacus Concepts, Berkley, CA, USA). *P* values less than 0.05 were accepted as statistically significant.

3. Results

3.1. Auditory brainstem response thresholds

There was no difference between the auditory brainstem response thresholds of treated and control ears before noise exposure (data not shown). Implantation of the osmotic pump and intracochlear infusion of saline had little influence on the auditory brainstem response threshold. In all animals, the

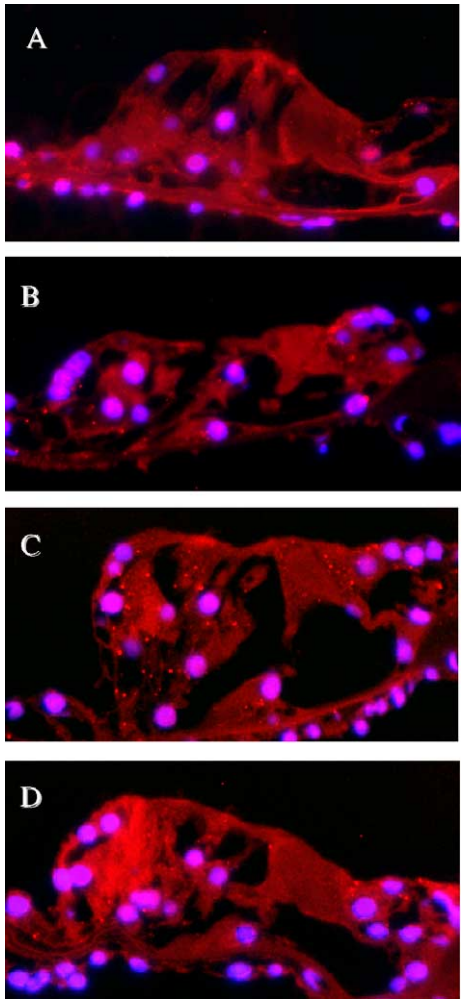

Fig. 3. Immunoreactivity to 4-hydroxy-2-nonenal (4-HNE) (red) in the organ of Corti following noise exposure. Nuclei were visualized with DAPI counterstaining (blue). Sections were taken from an area of the main lesion of the second turn of the cochlea. (A) Control; (B) immediately after noise exposure; (C) 9 h after noise exposure; (D) 21 h after noise exposure. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

auditory brainstem response thresholds of both ears were increased 7 days after noise exposure (Fig. 1). However, the threshold shift was smaller in the treated ears than in the control ears, in the −9, +9 and +21 h groups. In the +33 h group, there was little difference between the thresholds of the treated and control ears. Of the treated ears, the threshold shift was the smallest in the +9 h group.

3.2. Outer hair cell counts

After noise exposure, all animals showed a loss of outer hair cells in the second turn of the cochlea. The inner hair cells of all ears were relatively well preserved (data not shown). In the −9, +9 and +21 h groups, the percentage of missing outer hair cells was lower in the edaravone-treated ears than in the control ears, but in the +33 h group, there was no significant difference between the treated and control ears (Fig. 2). The smallest percentage of lost hair cells was observed in the edaravone-treated ears in the +9 h group.

3.3. Immunohistochemistry for 4-HNE

All sections for 4-HNE assessment were taken from the main lesion of the second turn (approximately 9–12 mm from the apex). Staining was absent from negative control material tested without the primary antibody (not shown). Slight staining was observed in the organ of Corti and the stria vascularis of unexposed control animals (Figs. 3A, 4A). Organ of Corti sections from animals killed immediately after noise exposure (Fig. 3B) showed little immunoreactivity. However, sections from animals killed 9 h after noise exposure (Fig. 3C) showed immunoreactivity against 4-HNE in some cells of the organ of Corti. Increased HNE staining was seen in the region of the Hensen cells and Claudius cells 21 h after noise exposure (Fig. 3D). Stria vascularis sections from animals killed immediately after noise exposure (Fig. 4B) showed 4-HNE immunoreactivity, and the extent of specific immunoreactivity increased with time (Fig. 4C, D). In the spiral ganglion, 4-HNE immunoreactivity at each time point did not differ significantly from that of controls (not shown).

4. Discussion

In the present study, edaravone did not provide sufficient cochlear protection in the −9 h group, which received edaravone starting 9 h before noise exposure; however, less cochlear damage was observed in the +9 and +21 h groups, which received edaravone beginning 9 and 21 h, respectively, after noise exposure. One reason for this discrepancy may be the stability of edaravone in the pumps. The pharmacological action of edaravone gradually decreases after edaravone is dissolved in saline; however, its efficacy is reported to be stable up to 24 h after edaravone is dissolved (Mitsubishi Pharma Co.; personal communication). If edaravone is effective 24 h after it is dissolved, edaravone administered between 12 and 24 h after pump exchange should be effective. In the −9 h group, edaravone was administered until the end of noise exposure. In the +9, +21 and +33 h groups, edaravone was administered until 21, 33 and 45 h, respectively, after noise exposure. It is also possible that most free radical formation occurred several hours after noise exposure. Immunohistochemical analysis of the organ of Corti revealed that 4-HNE did not form immediately after noise exposure. 4-HNE was first detected 9 h after noise exposure and staining was increased 21 h after noise exposure. Yamashita et al. (2004) also reported the delayed formation of 4-HNE in the organ of Corti

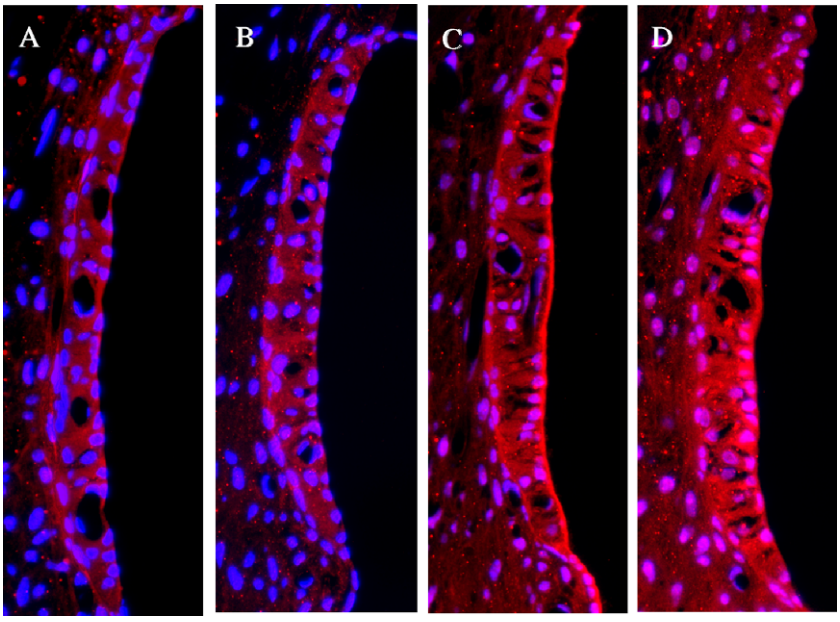

Fig. 4. Immunoreactivity to 4-HNE (red) in the stria vascularis following noise exposure. Nuclei were visualized with DAPI counterstaining (blue). Sections were taken from the main lesion of the second turn of the cochlea. (A) Control; (B) immediately after noise exposure; (C) 9 h after noise exposure; (D) 21 h after noise exposure. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

following noise exposure. Therefore, we speculate that the main damage in the organ of Corti occurs between 9 and 21 h after noise exposure. Edaravone-treated ears of the +9 h group showed the least damage.

The causes of noise-induced hearing loss remain unclear. One of the many mechanisms by which sound may damage the cochlea is through cochlear ischemia and reperfusion injury. Reduced cochlear blood flow and/or intracochlear oxygen levels (Thorne and Nuttall, 1987; Lamm and Arnold, 1998, 2000; Miller et al., 2003) and vasoconstriction of cochlear capillaries (Hawkins, 1971) in response to noise have been reported. These findings suggest that ischemia and subsequent reperfusion are important pathophysiological processes in noise-induced hearing loss. Reperfusion is known to cause extensive tissue injury. Kaminski et al. (2002) reported that reactive oxygen species and neutrophils interact with each other and are the key players in reperfusion injury. Activated neutrophils migrate from the vascular compartment to ischemic tissue and are one of the main sources of reactive oxygen species during reperfusion. In this study, 4-HNE was first detected in the organ of Corti, followed by the stria vascularis. During ischemia, neutrophils remained and reactive oxygen species were generated in the lumen of the stria vascularis because of vasoconstriction of cochlear capillaries. After the restoration of blood flow, however, neutrophils migrated from the stria vascularis to ischemic tissue, such as the organ of Corti, and reactive oxygen species were generated in the organ of Corti. Lamm and Arnold (1998, 2000) showed that cochlear blood flow and perilymphatic oxygen tension declined gradually during 30 min of noise exposure and for 180 min thereafter. Therefore, it is thought that ischemia was prolonged and the start of reperfusion was delayed, although noise did not cause complete ischemia. Additionally, Albertine et al. (1994) showed that ischemia-reperfusion causes a three-fold increase in neutrophil numbers in the lumen of arterioles and venules by 60 min of reperfusion and up to a seven-fold increase by 270 min of reperfusion. In this study, 4-HNE staining was observed in the supporting cells, not the outer hair cells, within 21 h, and it seems that the immunostaining spreads to the outer hair cells 21 h later. Yamashita et al. (2004) reported that immunostaining had spread to all cells of the organ of Corti at 7 days after the noise exposure. These reports and our results demonstrate that post-exposure administration of edaravone is important because the events that lead to the generation of reactive oxygen species, such as reperfusion and infiltration by neutrophils, occur well after noise exposure.

Our osmotic pump method (Sugahara et al., 2001) allows continuous microinjection into the inner ear, so the effect of edaravone is enhanced. Several drug-delivering systems are used clinically. One method of delivery is to place edaravone-soaked Gelfoam on the round window; this does not damage the cochlea (Horiike et al., 2003, 2004). Intratympanic drug injection is also a possible means of drug delivery (Doyle et al., 2004).

We conclude that topical administration of edaravone may prevent noise-induced hearing loss and that edaravone treatment will be clinically effective, especially if given within 21 h of noise exposure.

Acknowledgments

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