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Oral administration of geranylgeranylacetone to protect vestibular hair cells

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ABSTRACT

Objective: We recently reported that the heat shock response played a major role in the protection of hair cells against stress. Oral administration of the heat shock inducer, geranylgeranylacetone (GGA) protected hair cells against intense noise. In our present study, we investigated the effect of GGA on vestibular hair cell death induced by an aminoglycoside.

Methods: We used CBA/N mice aged 4–6 weeks. The mice were divided into two groups, GGA and control. Mice in the GGA group were fed a diet containing GGA (0.5%) for 4 weeks, and those in the control group were fed a standard diet. Immunohistochemical analyses for Hsp70 were performed in four animals. The utricles of the remaining animals were cultured in medium for 24 h with neomycin to induce hair cell death. After fixation, the vestibular hair cells were immunohistochemically stained against calmodulin, and hair cell survival was evaluated.

Results: The vestibular hair cells of mice in the GGA group expressed Hsp70. In addition, after exposure to neomycin, vestibular hair cell survival was higher in the GGA group than in the control group.

Conclusion: Our results demonstrated the oral administration of GGA induced the heat shock response in the vestibule and could protect sensory cells.

1. Introduction

Sensory hair cells are easily damaged by exposure to aminoglycosides, infection, and ischemia [1]. After the hair cells are damaged, auditory and vestibular dysfunction is permanent in mammalian; therefore, it is important to prevent the loss of hair cells in patients with inner ear diseases.

Heat shock proteins (HSPs) are induced in cells as a protective mechanism against cellular stress. The activation of the heat shock response is mediated by heat shock transcription factors (HSFs). Recent studies have revealed the role of HSPs and HSFs in the maintenance mechanism of mammalian sensory organs [2,3]. The anti-ulcer drug geranylgeranylacetone (GGA) has been reported to activate HSF1 and induce HSP expression [4]. Administration of GGA confers a protective effect that is associated with HSP upregulation in various organs such as the gastric mucosa, small intestine, spinal nerves, liver, heart, brain, and retina [5]. In relation to the inner ear, we previously showed that GGA administration could prevent hair cell loss and hearing impairment from noise exposure in guinea pigs, and attenuated progressive hearing loss in a model of age-related hearing loss via induction of major HSPs [6,7].

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In the present study, we used cultured utricle systems to investigate the protective effect of the oral administration of GGA against hair cell degeneration induced by neomycin.

2. Materials and methods

2.1. Animal use and care

CBA/N male mice obtained from Kyushu Animal Company (Kumamoto, Japan) were used in this study. All mice were 4- to 6-week-old, and had normal Preyer's reflexes. The experimental protocol was reviewed and approved by the Committee for Ethics on Animal Experiments at the Yamaguchi University School of Medicine, and experiments were conducted in accordance with these guidelines, Japanese federal law (No. 105), and Notification No. 6 of the Japanese government.

2.2. Oral administration of GGA

GGA granules (Selbex®; Eisai, Tokyo, Japan) were mixed with powdered rodent feed at a concentration of 0.5%, which corresponded to a dose of 400–600 mg/kg/day during the study [8]. In the GGA group, GGA was administered for a period of 30 days prior to dissection. In the control group, the animals were fed a normal powdered rodent feed.

2.3. RT-PCR of Hsp70 expression

Four mice in each group were deeply anesthetized with an overdose of pentobarbital and immediately decapitated. The temporal bones were immediately dissected and frozen in liquid nitrogen. Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA), and cDNA was synthesized from 2 μg of total RNA using a cloned MV first-strand cDNA synthesis kit that contained avian myeloblastosis virus-reverse transcriptase, oligo(dT) 20, and a random hexamer (Invitrogen). Thirty cycles of PCR were performed using Ex Taq polymerase (Takara, Japan) and 1 μL from each reverse transcription product. The following primers were used for amplification: Hsp70, (5′-AACAAGATCACCATCAC-3′, 5′-TCCACCTCCTCGATGGT-3′); β-actin, (5′-CATGTACGTTGCTATCCAGGC-3′, 5′-CTCCTTAATGTCACGCACGAT-3′). The amplified DNA fragments were resolved using agarose gel electrophoresis, stained with ethidium bromide, and photographed using an Epi-Light UV FA1100 (Asian Cosmos, Japan). mRNA levels were estimated by densitometry of the bands using the public domain image processing program ImageJ (http://rsbweb.nih.gov/ij/download.html).

2.4. Organ culture of utricles and induction of hair cell death

All animals were deeply anesthetized with an overdose of pentobarbital and immediately decapitated. The temporal bones were quickly removed and the individual vestibular organs were dissected in basal Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with Earle's balanced salt solution (Invitrogen) (2:1, v/v). Isolated utricles were transferred into the culture medium, which consisted of basal Eagle's medium supplemented with Earle's balanced salt solution (2:1, v/v) and 5% fetal bovine serum (Invitrogen). The free-floating utricles were incubated in 24-well tissue culture plates for 24 h at 37 °C in an atmosphere of 5% CO_2 and 95% air. To induce hair cell death, neomycin solution (10 mg/mL; Sigma, St. Louis, MO) was added into the culture wells to give a final concentration of 1.0 mM. After the culture protocols were completed, the utricles were fixed with 4% paraformaldehyde (PFA) for 1 h at room temperature. Otoconia were gently removed from the fixed utricles by a stream of phosphate buffered saline (PBS) applied via a 28-G needle and syringe. Before use in the assays outlined below, the samples were rinsed with PBS.

2.5. Immunohistochemical staining of Hsp70

To evaluate the induction of the heat shock response, the expression of Hsp70 was investigated. After incubating with blocking solution (1% bovine serum albumin, 0.4% normal goat serum, 0.4% normal horse serum, and 0.4% Triton X-100 in PBS), the specimens obtained by the methods described above were incubated with an antibody against Hsp70 [9] (1:100 dilution) for 12 h at 4 °C. Then, after washing in blocking solution, the specimens were incubated with secondary antibodies diluted in blocking solution: Alexa 488-conjugated goat anti-mouse IgG (1:500) and Alexa 594-conjugated goat anti-rabbit IgG (1:500), which were both obtained from Molecular Probes, Eugene, OR. The utricles were rinsed with blocking solution, mounted in Vectashield® (Vector Laboratories), and coverslipped. The samples were observed with a fluorescence microscope (BZ-8100, KEYENCE, Osaka, Japan). The fluorescence intensity of the immunohistochemistry against Hsp70 was evaluated with ImageJ. The average of the fluorescence intensity derived from the cultured utricles was presented as a relative value to the control. Six samples from GGA group were compared with 6 samples from control group in the experiment.

2.6. Immunohistochemistry for hair cell labeling

Fixed utricles were incubated in blocking solution overnight at 4 °C. To label the hair cells, a monoclonal antibody against calmodulin (1:150 dilution; Sigma) and a polyclonal antibody against calbindin (1:250 dilution; Chemicon, Temecula, CA) were used. The specimens were incubated overnight at 4 °C in the primary antibody solution, washed in blocking solution, and incubated with secondary antibodies diluted in blocking solution as detailed above. The utricles were rinsed with blocking solution, mounted in Vectashield®, and coverslipped for microscopy.

2.7. Evaluation of the number of residual sensory hair cells

Utricles were examined under a BZ-8100 fluorescence microscope to evaluate hair cell survival. Calbindin-positive and calmodulin-positive cells were counted in the striolar region and extrastriolar region, respectively. The labeled hair cells were counted in two 20 μm × 20 μm squares that were

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