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Full paper

Involvement of calpain in 4-hydroxynonenal-induced disruption of gap junction-mediated intercellular communication among fibrocytes in primary cultures derived from the cochlear spiral ligament

Taro Yamaguchi [a, b], Masanori Yoneyama [a], Eiichi Hinoi [b], Kiyokazu Ogita [a, *]

[a] Laboratory of Pharmacology, Faculty of Pharmaceutical Sciences, Setsunan University, Hirakata, Osaka 573-0101, Japan
[b] Laboratory of Molecular Pharmacology, Graduate School of Natural Science and Technology, Kanazawa University, Kanazawa, Ishikawa 920-0934, Japan

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ABSTRACT

The endocochlear potential in the inner ear is essential for hearing ability, and maintained by various K^+ transport apparatuses including Na^+, K^+-ATPase and gap junction-mediated intercellular communication (GJ-IC) in the lateral wall structures of the cochlea. Noise-induced hearing loss is known at least in part due to disruption of GJ-IC resulting from an oxidative stress-induced decrease in connexins (Cxs) level in the lateral wall structures. The purpose of this study was to investigate, using primary cultures of fibrocytes from the cochlear spiral ligament of mice, the mechanism underlying GJ-IC disruption induced by 4-hydroxynonenal (4-HNE), which is formed as a mediator of oxidative stress. An exposure to 4-HNE produced the following events: i.e., an increase in 4-HNE-adducted proteins; a decrease in the protein levels of Cx43, β-catenin, and Cx43/β-catenin complex along with intracellular translocation of this complex from the cell membrane to the cytoplasm; enhanced calpain-dependent degradation of endogenous α-fodrin; and disruption of GJ-IC. The 4-HNE-induced decrease in these protein levels and disruption of GJ-IC were most completely abolished by the calpain inhibitor PD150606. Taken together, our data suggest that 4-HNE disrupted GJ-IC through calpain-mediated degradation of Cx43 and β-catenin in primary cultures of fibrocytes derived from the cochlear spiral ligament.

1. Introduction

Spiral ligament fibrocytes (SLFs) of cochlear lateral wall structures play crucial roles in auditory function in the mammalian cochlea. These roles include K^+ homeostasis in the cochlea to generate the endocochlear potential, which is essential for depolarization of cochlear hair cells for sound transduction, and maintained by various K^+ transport apparatuses including Na^+, K^+-ATPase and gap junction (GJ)-mediated intercellular communication (GJ-IC) in the lateral wall structures of the cochlea (1–3). Once hair cells are activated by sound, the endocochlear potentials are generated by the flow of K^+ from the endolymph into the hair cells. It has been postulated that a K^+ recycling pathway toward the stria vascularis via the SLFs in the cochlear lateral wall structures is critical for proper hearing, although its exact mechanism has not been definitively determined (2). Accumulating evidence demonstrated that intense noise-induced hearing loss is at least in part due to the abnormal endocochlear potentials induced by dysfunction of the stria vascularis and spiral ligament in the cochlear lateral wall structures (4, 5). However, the mechanism underlying the noise-induced dysfunction of the lateral wall structures is not fully understood.

GJ-IC is known to play an important role in maintaining the unique ionic composition of the endolymph and intracellular ion content, which are crucial for cochlear functions. The GJ is an intercellular membrane channel that has the unique feature of directly connecting the cytoplasm of neighboring cells. It is formed by the juxtaposition of 2 hexameric structures (termed hemichannels or connexons) formed from connexins (Cxs) at the GJ plaques, where a large number of GJs are clustered at the cell–cell contact points. Non-sensory cells in the cochlea are connected extensively by GJs that facilitate intercellular ionic and biochemical

* Corresponding author. Laboratory of Pharmacology, Faculty of Pharmaceutical Sciences, Setsunan University, 45-1 Nagaotoge-cho, Hirakata, Osaka 573-0101, Japan. Tel./fax: +81 (0)72 866 3110.
E-mail address: ogita@pharm.setsunan.ac.jp (K. Ogita).
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coupling for the GJ-IC. Several different Cx subunits have been reported to be expressed in the mammalian inner ear. These include Cx26, Cx30, Cx31, and Cx43 (6–9). Evidence for the involvement of Cxs in hearing ability comes from the finding that mutations in the Cx26-encoding gene occur in a substantial portion (20–50%) of human non-syndromic hereditary deafness cases, which type of deafness is one of the most common human birth defects. A large number of reports on human GJB2 mutations linked to prelingual deafness has described loss-of-function mutations that effectively nullify the utility of Cx26 in the cochlea (10). Disturbance of the GJ complex of Cxs is expected to disrupt the recycling of K^+ from the synapses at the base of the hair cells through the supporting cells and SLFs in the cochlea. In addition, the disruption of K^+ recycling is known to decrease sound-induced cochlear responses, resulting in sensorineural hearing loss (11). Our previous report indicated that exposure to noise *in vivo* produced a mediator of oxidative stress, 4-hydroxynonenal (4-HNE), in the cochlear lateral wall structures (12) and that disruption of GJ-IC by it involves a decrease in Cx levels in the lateral wall structures (13). The purpose of this study was to investigate the mechanism underlying GJ-IC disruption induced by 4-HNE by using primary cultures of cochlear SLFs.

2. Materials and methods

2.1. Materials

4-HNE was purchased from Cayman Chemical (Ann Arbor, MI. USA). 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) and PD150606 were supplied from Sigma–Aldrich Co. (St. Louis, MO, U.S.A.). Mouse monoclonal antibodies against Cx43 and α-fodrin were obtained from Zymed Laboratories, Inc. (South San Francisco, CA, USA) and Biomol International, Inc. (Plymouth Meeting, PA, USA), respectively. Rabbit polyclonal antibodies against β-catenin, 4-HNE, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Sigma–Aldrich Co. (St. Louis, MO, U.S.A.), Calbiochem, Inc. (La Jolla, CA, USA), and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), respectively. Alexa-Fluor 568-conjugated anti-rabbit IgG (H + L) antibody, Alexa-Fluor 488-conjugated anti-mouse IgG (H + L) antibody, calcein-AM, and 1,1-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchloric acid (DiI) were purchased from Life Technology, Co. (Carlsbad, CA, USA). Poly-L-lysine hydrobromide and trypsin solution were from Nacalai Tesque, Inc. (Kyoto, Japan). Polyvinylidene fluoride membranes (Immobilon-P) were obtained from Millipore (Bedford, MA, USA). Western Lightning Chemoluminescence Reagent Plus was purchased from PerkinElm1er Life Science Products, Inc. (Boston, MA, U.S.A.). NeutrAvidin UltraLink Resin and EZ-Link Sulfo-NHS-Biotin were from Thermo Scientific, Inc. (Waltham, MA, USA). Protein G Sepharose was supplied from GE Health Care, Inc. (Waukesha, WI, USA). Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Wako, Inc. (Osaka, Japan). All other chemicals used were of the highest purity commercially available.

2.2. Animal treatment

The protocol used here met the guidelines of the Japanese Society for Pharmacology and was approved by the Committee for Ethical Use of Experimental Animals at Setsunan University. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to *in vivo* techniques. Adult male Std-ddY mice weighing 26–28 *g*, which we routinely use for neuroscience studies, were housed in metallic breeding cages in a room with a light–dark cycle of 12 h–12 h and a humidity of 55% at 23 °C and given free access to food and water. To avoid the use of animals with natural auditory impairment, we measured their ABR before use and selected those animals with normal acoustic sense in the present study.

2.3. Primary cultures of cochlear SLFs

Cultured SLFs were prepared from the cochlear spiral ligament of intact mice according to our previous study (13). Briefly, mouse cochleae were removed from 5 animals under sterile conditions and transferred to ice-cold Dulbecco's phosphate-buffered saline containing 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 33 mM glucose. Following opening of the cochlear bone, the spiral ligament tissues were dissected with fine forceps and cut into small longitudinal tissue segments that were put into Petri dishes (35 mm) pre-coated with type 1 collagen and containing 0.3 mL culture medium consisting of DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 0.1 mg/mL streptomycin. At 3 and 4 days *in vitro* (DIV), 0.7 mL and 1 mL, respectively, of fresh culture medium was added to each Petri dish. The tissue segments were further cultured for 6 days with the culture medium being changed every 3 days. During the culture period, fibrocytes migrated from the tissue segments and reached confluence at 10 DIV. These cells were rinsed with phosphate-buffered saline and incubated with 0.25% trypsin solution for 5 min at 37 °C to induce cell detachment. Fresh culture medium was then added and gently triturated to suspend the cells. After centrifugation at 900× *g* for 5 min, the cells thus obtained were washed twice with culture medium by gentle trituration and centrifugation. Then the cells were seeded at a density of 1000 cells/0.5 mL in each well of 4-well dishes (Nunc, Denmark) that had been previously coated with poly-L-lysine hydrobromide and then cultured for the desired periods with a change of the culture medium every 3 days. The cells were usually used for experiments at 12 DIV. The cultures were always maintained at 37 °C in 95% (vol/vol) air −5% (vol/vol) CO_2.

2.4. Immunostaining of cultures

Fibrocytes were fixed with 4% (wt/vol) paraformaldehyde for 20 min at 4 °C. After having been blocked for 1 h at room temperature with 5% (vol/vol) goat serum in Tris-buffered saline (pH 7.5) containing 0.03% (wt/vol) Tween 20 (0.03% TBST), the cells were incubated at 4 °C overnight with primary antibody against 4-HNE (1:200), Cx43 (1:100) or β-catenin (1:300). After a wash with 0.03% TBST, they were then reacted with fluorescence-labeled secondary antibodies (Alexa488–conjugated anti-mouse IgG [1:200] for Cx43; Alexa568-conjugated anti-rabbit IgG [1:200] for 4-HNE and β-catenin) for 2 h at room temperature. Finally, they were counterstained with DAPI (1 μg/mL) for 20 min at room temperature. Stained cells were observed by confocal laser scanning microscopy (FV1000-D, Olympus, Tokyo).

2.5. Immunoblot analysis

The cells were quickly removed and immersed in ice-cold homogenizing buffer consisting of 10 mM Tris–HCl buffer (pH 7.5), 0.32 M sucrose, 1 mM EDTA, 1 mM EGTA, 5 mM dithiothreitol, 1% Triton-X100, phosphatase inhibitors (10 mM sodium β-glycerophosphate and 1 mM sodium orthovanadate), and 1 μg/mL of each of the following protease inhibitors: (p-amidinophenyl)methanesulfonyl fluoride, benzamidine, leupeptin, and antipain. After homogenization in 30 μL of the homogenizing buffer, the cellular lysates were immediately boiled for 10 min in a solution comprising 2% (wt/vol) SDS, 5% (vol/vol) 2-mercaptoethanol, 10% (vol/vol) glycerol, and 0.01% (wt/vol) bromophenol blue immediately. The samples were stored at −35 °C until used for immunoblot analysis.