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Proteasome inhibitors induce auditory hair cell death through peroxisome dysfunction

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ABSTRACT

Even though bortezomib, a proteasome inhibitor, is a powerful chemotherapeutic agent used to treat multiple myeloma (MM) and other lymphoma cells, recent clinical reports suggest that the proteasome inhibitor therapy may be associated with severe bilateral hearing loss. We herein investigated the adverse effect of proteasome inhibitor on auditory hair cells. Treatment of a proteasome inhibitor destroys stereocilia bundles of hair cells resulting in the disarray of stereocilia in the organ of Corti explants. Since proteasome activity may be potentially important for biogenesis and function of the peroxisome, we tested whether proteasome activity is necessary for maintaining functional peroxisomes. Our results showed that treatment of a proteasome inhibitor significantly decreases both the number of peroxisomes and expression of peroxisomal proteins such as PMP70 and Catalase. In addition, we also found that proteasome inhibitor impairs the import pathway of PTS1-peroxisome matrix proteins. Taken together, our findings support recent clinical reports of hearing loss associated with proteasome inhibition. Mechanistically, peroxisome dysfunction may contribute to hair cell damage and hearing loss in response to the treatment of a proteasome inhibitor.

1. Introduction

Peroxisomes contain more than 100 enzymes and other proteins to conduct cellular metabolic functions including very long and branched chain fatty acid oxidation, synthesis of bile acids and plasmalogen, conversion of hydrogen peroxide to nontoxic forms [1,2]. Mutations in genes involved in peroxisome biogenesis or peroxisomal function cause inheritable genetic disorders, such as Zellweger syndrome, infantile Refsum disease, neonatal adrenoleukodystrophy [3–5]. Peroxisomes are derived from the ER and undergo maturation by importing peroxisome matrix proteins from the cytosol through the specific peroxisome target sequence (PTS) [6]. For importing the matrix proteins into the peroxisome, several Pex proteins are participated in the process of peroxisome assembly. Among them, Pex2, 10, 12 are E3 ubiquitin ligase, and Pex5 acting as PTS receptor is subject to poly-ubiquitination and degradation by proteasome during peroxisome formation [7]. These findings suggested that proteasome function might be important for biogenesis and function of the peroxisome.

The ubiquitin-proteasome pathway plays an important role for intracellular protein degradation to regulate various cellular processes. The ubiquitin conjugating system is composed of ubiquitin activating enzymes (E1), ubiquitin conjugating enzymes (E2), ubiquitin ligases (E3), de-ubiquitinases and the 26S proteasome-containing multi-protease complex [8,9]. Inhibition of the proteasome function affects the active forms of proteins that regulate cell cycle progression, transcription, inflammation, apoptosis, and angiogenesis [10,11]. Bortezomib acting as proteasome inhibitor is an anticancer agent used to treat multiple myelolma (MM) [12]. However, recent reports showed that bortezomib therapy is associated with hearing loss [13,14]. Notably, a sensory deafness has been known as one of the clinical symptoms associated with peroxisome defects, such as Zellweger syndrome, infantile Refsum disease, acyl-CoA Oxidase deficiency, and D-bifunctional protein deficiency [15–18]. Therefore, we investigated a potential link between proteasome inhibition and peroxisome dysfunction which may result in hair cell damage and hearing loss.

2. Materials and methods

2.1. Cell culture and viability

Establishment and characterization of conditionally immortalized auditory HEI-OC1 cells have well been described. Cells were

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cultured in high-glucose DMEM (GIBCO BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; GIBCO BRL) at 33 °C and 5% CO_2 in humidified atmosphere. Cells were grown in a humidified atmosphere at 37 °C, 5% CO_2. To determine cell viability, cells were treated with MTT (0.5 mg) for 2 h and were washed three times with phosphate buffered saline (PBS, pH 7.4). Insoluble formazan product was dissolved in DMSO and the optical density (OD) of each culture well was measured using a microplate reader (Titertek Multiskan, Flow Laboratories) at 590 nm. The OD of the control cells was taken as 100% viability.

2.2. Organotypic cultures of Corti's organ explants

The organotypic culture procedure was described previously [19]. In short, Sprague Dawley rats were sacrificed on postnatal day two (P3) and the cochlea were carefully dissected. The stria vascularis and the spiral ligament were dissected away leaving the organ of Corti. For further study, the organ of Corti was dissected into three regions of the apical, middle and basal turns. Cochlear explants were treated with DMEM containing glucose (4.5 g/l) and 10% FBS during the stationary phase of cell growth at 12 h post-dissection. Cochlear explants were treated with 5 μM MG132 (Sigma) and bortezomib (Santa Cruz), then incubated for 30 h at 37 °C, 5% CO_2. The control group, maintained with serum containing DMEM medium only, was cultured concurrently with the experimental groups. At the end of the experiment, the cultures were prepared for histological analysis. Organotypic cultures of Corti's organ were fixed in 2% paraformaldehyde in PBS at RT for 20 min. The specimens were rinsed with PBS, then incubated in 0.25% Triton X-100 for 5 min, and immersed in tetramethyl rhodamine isothiocyanate (TRITC)-labeled phalloidin (Sigma, 1:100 diluted) in PBS for 20 min. After rinsing three times with PBS, the specimens were examined under a fluorescence microscope. This work was carried out in conformity with all applicable regulations and institutional use rules of Laboratory Animals in Wonkwang University School of Medicine.

2.3. Western blot analysis

Cells (3×10^6) were scraped off the culture plates and centrifuged at 1000×*g* for 5 min at 4 °C. Cells were homogenized with lysis buffer (10 mM Tris–HCl pH 7.6, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 1 mM EDTA, 2.5 mM b-glycerophosphate, 1 mM dithiothreitol, 1 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethanesulfonylfluoride, and 1× protease inhibitor cocktail) and centrifuged at 14,000 rpm for 10 min at 4 °C, and the supernatant was taken. Cell lysates were heated at 100 °C for 15 min and subject to SDS–PAGE electrophoresis. Separated proteins were electroblotted onto a nitrocellulose membrane. The membrane was then incubated in TBS-T blocking buffer for 1 h and carried out the antibody binding reaction for the first antibody and secondary HRP-conjugated antibody. Antibody against PMP70 was purchased from Thermo Scientific (Rockford, IL, USA). Anti-β-actin antibody was purchased from Santa Cruz Biotech Inc. (Santa Cruz, CA, USA). Anti-catalase antibody was purchased from Rockland (Gilbertsville, PA, USA). After extensive washes, protein bands on the membrane were visualized using an enhanced chemiluminescence detection system (Amersham, Piscataway, NJ, USA) according to the manufacturer's recommended protocol.

2.4. RT-PCR (Q-PCR)

Total RNAs were extracted from cultured cells with TRIzol reagent (Invitrogen). A reverse transcription kit (Roche, Indianapolis, IN) was used to construct the template cDNA for real-time PCR (Q-PCR) with LightCycler using FastStart DNA Master SYBR Green I (Roche, Indianapolis, IN). The primer sequences for mouse cDNAs were as follows:

PMP70-1: 5′-gggagaagcagacaatccac-3′ and reverse, 5′-ccgaaagaa aatgaaattatgtagg-3′, Catalase: forward, 5′-cttcaagttggttaatgcaga-3′ and reverse, 5′-caagtttttgatgccctggt-3′. Pex2: forward, 5′-tga aggaaccacttagaaattacaga-3′ and reverse, 5′-ccagggccttattcagttca-3′. Pex14: forward, 5′-ctcactccgcagccataca-3′ and reverse, 5′-agccaaggcaccataatctc-3′. Pex11α: forward, 5′-ttcatccgagtcgccaac-3′ and reverse, 5′-catgcatgcgtgctgagt-3′. Pex11β: forward, 5′-gagcctc ggacgaaagttg-3′ and reverse, 5′-caggtgcacagccctttt-3′. ACOX1: forward, 5′-agattggtagaaattgctgcaaaa-3′ and reverse, 5′-acgccactt ccttgctcttc-3′. HSD17B4: forward, 5′-gggagcagtacttggagctg-3′ and reverse, 5′-tcagcaataactgcttcacatttt-3′. SCPx: forward, 5′-ggttggct atgatatgagtaaagaagct-3′ and reverse, 5′-agctctatcacgtcgacatcgtt-3′. EHHADH: forward, 5′-ccggtcaatgccatcagt-3′ and reverse, 5′-ctaaccgt atggtccaaactagc-3′. PTHlO: forward, 5′-agagactgcctgactcctatgg-3′ and reverse, 5′-ctgcttctgccgtgaaatg-3′. Internal control 36B4: 5′-cact ggtctaggacccgagaag-3′; and reverse, 5′-ggtgcctctggagattttcg-3′. Each analysis was performed in triplicate.

2.5. Immunofluorescence

Cells were washed with PBS, fixed in 4% paraformaldehyde (Sigma–Aldrich HT5014) at room temperature for 20 min, rinsed in PBS, incubated in 0.1% Triton X-100, and then rinsed in PBS. After incubation in 3% BSA (Bovine Serum Albumin), cells were incubated with 1:400 of target protein antibody at 4 °C. Next, plates were carefully washed with PBS, stained with Alexa flur-568 dye, washed twice with PBS and incubated with 10 μM DAPI in PBS at RT for 30 min. After mounting the coverslip, cells were observed under a fluorescence microscope.

2.6. Preparation of cytosol and membrane fraction tractions

Cells were washed with ice-cold PBS, scraped and centrifuged at 1000×*g* for 5 min at 4 °C. The cell pellet was then homogenated in 400 μl of buffer (10 mM HEPES, pH 7.9, 50 mM NaCl, 0.1 mM EDTA, 0.25 M Sucrose). The homogenates were centrifuged at 1000×*g* for 10 min at 4 °C and the supernatant was transferred a new tube. The supernatant was centrifuged at 13,000×*g* for 30 min at 4 °C. After centrifugation, the supernatant (cytosol fraction) was transferred to a new tube and the pellet (membrane fraction) was resuspended in lysis buffer. Both fractions were used for Western blot analysis.

2.7. Generation of stably transfected HEI-OC1 cells expression RFP-SKL

HEI-CO1 cells were transfected with 2 μg of pRFP-SKL plasmid using X-tremeGENE9 DNA transfection reagent (Roche). 24 h after transfection, cells were switched to a medium supplemented with 700 μg/ml G418 to select for cells expressing a neomycin-containing plasmid. Fresh medium was added every 2–3 days until colonies were formed at ~15 days. Individual colonies were isolated with cloning cylinders, and expression of RFP-SKL was assessed under a fluorescence microscope.

3. Results

To investigate the ototoxic effect of proteasome inhibitor, HEI-OC1 auditory cells were treated for 24 h with various concentrations of MG132, a proteasome inhibitor. As shown in Fig. 1A, MG132 decreased cell viability in a dose-dependent manner. To verify the result from HEI-OC1 cells, we examine the effect of MG132 on sensory hair cells in the primary cultures of organ of Corti isolated from postnatal day 3 (P3) rat. The organ of Corti was treated with 5 μM MG132 for 24 h. After incubation, F-actin