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The influence of sphingosine-1-phosphate receptor antagonists on gentamicin-induced hair cell loss of the rat cochlea

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HIGHLIGHTS

- Sphingosine-1-phosphate (S1P) receptors 1–3 ($S1PR_{1-3}$) were expressed in the organ of Corti and spiral ganglion.
- An $S1PR_2$ antagonist increased gentamicin-induced hair cell loss.
- These results indicate a possibility that S1P act as a cochlear protectant against gentamicin ototoxicity via activation of $S1PR_2$.

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ABSTRACT

Sphingosine-1-phosphate (S1P) is a sphingolipid metabolite that regulates various critical biological processes, such as cell proliferation, survival, migration, and angiogenesis. The action of S1P is exerted by its binding to 5 specific G protein-coupled S1P receptors (S1PR), $S1PR_1$–$S1PR_5$. Aminoglycoside antibiotics including gentamicin induce cochlear hair cell loss and sensorineural hearing loss. Apoptotic cell death is considered to play a key role in this type of cochlear injury. S1P acts as a cochlear protectant against gentamicin ototoxicity. In the present study, expression of S1PRs in the cochlea was examined. In addition, the effects of S1PR antagonists on gentamicin ototoxicity were investigated using tissue culture techniques. Cochleas were dissected from Sprague-Dawley rats on postnatal days 3–5. Basal turn organ of Corti explants were exposed to 35 μM gentamicin for 48 h with or without S1PR antagonists. $S1PR_{1-3}$ were expressed in the organ of Corti and spiral ganglion. The $S1PR_2$ antagonist increased gentamicin-induced hair cell loss, while the $S1PR_1$ and $S1PR_3$ antagonists did not affect gentamicin ototoxicity. These results indicate the possibility that S1P act as a cochlear protectant against gentamicin ototoxicity via activation of $S1PR_2$.

1. Introduction

Sphingolipid metabolites, such as ceramides and sphingoid bases, have been implicated in the modulation of membrane signal transduction systems and in diverse cellular processes, such as cell proliferation, survival, migration, and angiogenesis [1–4]. We recently reported that exogenously applied sphingosine-1-phosphate (S1P) protected cochlear hair cells against gentamicin ototoxicity [5]. S1P exerts its cellular responses through a family of 5 G-protein-coupled S1P receptors (S1PRs) known as $S1PR_{1-5}$ [6–9]. These S1PRs are differentially expressed in various cell types. $S1PR_1$, $S1PR_2$, and $S1PR_3$ are widely expressed in cells and tissues, whereas $S1PR_4$ and $S1PR_5$ are expressed only in the cells of the immune and nervous systems [10]. Presence of $S1PR_{1-3}$ has been shown in the organ of Corti [11,12], but expression of $S1PR_{4,5}$ has never been examined before.

Aminoglycoside antibiotics are widely used for the treatment of infectious diseases. However, the clinical usage of aminoglycosides has often been limited owing to their side effects – ototoxicity and nepherotoxicity. Aminoglycosides are well known to damage cochlear inner ear hair cells, causing sensorineural hearing loss and balance disturbance [12]. Recent findings have demonstrated that death of cochlear hair cells was elicited by gentamicin via an apoptotic pathway, at least in part [13,14]. Particularly, evidence of the involvement of the intrinsic apoptotic pathway in gentamicin ototoxicity has been demonstrated [15].

The present study was designed to investigate the expression of S1PRs in the rat cochlea and to examine the role of S1PRs in hair cell death induced by gentamicin.

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2. Materials and methods

2.1. Animals

Postnatal days 3 (P3) to 5 (P5) Sprague-Dawley rats were used. All animal procedures were carried out according to the guidelines of the Laboratory Animal Research Center of Tsukuba University.

2.2. Reverse transcription-polymerase chain reaction (RT-PCR)

The cochlea, organ of Corti, and spiral ganglion were dissected. Total RNA was extracted from each cell, using Trizol (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed using total RNA (1 μg) with a GeneAmp PCR System 9600 (Perkin Elmer, Tokyo, Japan). The mRNA expression levels of $S1PR_{1-5}$ were detected by conventional RT-PCR with Taq polymerase (Takara, Shiga, Japan).

Glyceraldehyde-3-phoshate dehydrogenase (GAPDH) was used as an internal control for RNA integrity. S1PR primer sequences were as follows:

(1) $S1PR_1$-F: 5′-AGCGTTTGTCTGGAGAAGTACC-3′
$S1PR_1$-R: 5′-TAGCAAGGAGGCTGAAGACTGA-3′

(2) $S1PR_2$-F: 5′-CCTGAGAAGGTTCAGGAACACTAC-3′
$S1PR_2$-R: 5′-CCCAATGAGCATCAACATTCGAC-3′

(3) $S1PR_3$-F: 5′-ATGTCCGGTAGGAAGACGTTCA-3′
$S1PR_3$-R: 5′-AAGAAAGCACGCCGCATCTC-3′

(4) $S1PR_4$-F: 5′-GATCTTGGTGGCTTTTGTGG-3′
$S1PR_4$-R: 5′-CTCTCGCATCTTGAAGCTGA-3′

(5) $S1PR_5$-F: 5′-CCAGTGCACAAATGCCAA-3′
$S1PR_5$-R: 5′-GTTGTAGTGAAGGACGATGAC-3′

(6) GAPDH-F: 5′-AAGGTCATCCCAGAGCTGAA-3′
GAPDH-R: 5′-GTTGAAGTCACAGGAGACAACC-3′

2.3. Culture technique

The basal turn of the organ of Corti was dissected and cultured according to the methods of Van de Water and Ruben [16] and Sobkowicz et al. [17]. Cochlear explants were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 25 mM HEPES, and 30 U/mL penicillin. They were cultured in an incubator at 37 °C with 5% CO_2 at 95% humidity. Cochlear cultures were maintained in the above-described medium overnight (8–12 h) and then exposed to a medium containing 35 μM gentamicin for 48 h to assess the effects of S1PR antagonists [18,19]. Each S1PR antagonist was tested at concentrations of 1–100 μM.

2.4. S1P receptor antagonists

S1P and (R)-3-amino-(3-hexylphenylamino)-4-(oxobutyl-phosphonic) acid (W146, a selective $S1PR_1$ antagonist) and 1-[1,3-dimethyl-1-4-(2-methyllethyl)-1H-pyrazolo[3,4b]pyridin-6-yl]-4-(3,5-dichloro-4-pyridinyl)-semicar-bazide (JTE013, a selective $S1PR_2$ antagonist) were purchased from Sigma Japan (Tokyo, Japan). 2-Undecyl-thiazolidine-4-carboxylic acid (BML241, a selective $S1PR_3$ antagonist) was purchased from Cayman Chemical (Ann Arbor, MI, USA). W146 was initially dissolved in methanol to 10 mM and stored at −20 °C. JTE013 was initially dissolved in dimethyl sulfoxide (DMSO) to 10 mM and stored at −20 °C. BML241 was initially dissolved in dimethylformamide (DMF) to 10 mM and stored at −20 °C. Each antagonist was diluted in the culture medium to the final concentration immediately before use.

2.5. Cytochemistry

At the end of the tissue culture, the explants were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min and then permeabilized with 5% Triton X-100 (Sigma, St. Louis, MO, USA) in PBS with 10% fetal bovine serum (FBS) for 10 min. The specimens were stained with phalloidin with a conjugated Alexa Fluor probe (1:100, Molecular Probes, Carlsbad, CA, USA) at room temperature for 1 h. Phalloidin is a specific marker for cellular F-actin and labels stereociliary arrays and the cuticular plates of hair cells [12,18]. All experiments consisted of 8–20 explants per experimental group.

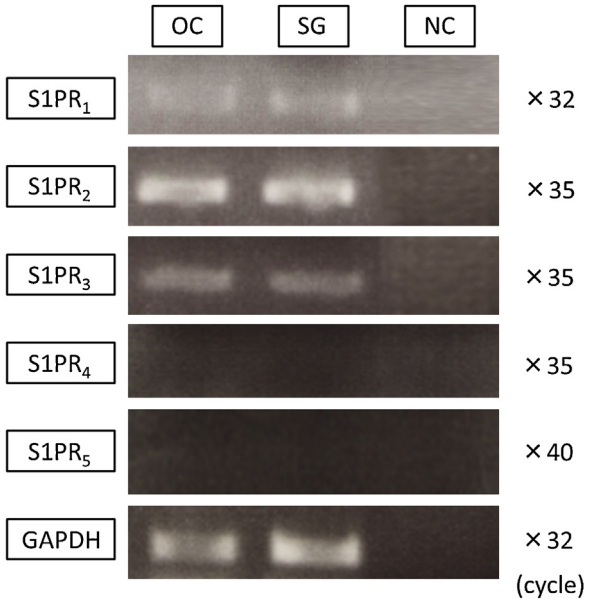

Fig. 1. Expression of S1PRs in the cochlea. RT-PCR analysis demonstrated that 3 S1P receptors ($S1P_{1-3}$) were expressed in the organ of Corti (OC) and spiral ganglion (SG) of Sprague-Dawley rats on postnatal days 3 (p3) to 5 (p5). However, the other 2 S1P receptors ($S1P_{4,5}$) were not detected. GAPDH primers served as a cDNA loading control (NC; negative control).

2.6. Assessment of cochlear hair cell damage

Hair cells were characterized as missing if no stereocilia or cuticular plates were observed by phalloidin staining. Quantitative results were obtained by evaluating 30 outer hair cells in a given microscopic field [15]. The average of 3 separate counts was used to represent each culture.

2.7. Western blot analysis

The organ of Corti and the spiral ganglion were homogenized in lysis buffer containing 0.25 M sucrose, 50 mM dithiothreitol, 3 mM HEPES (pH 7.9), 0.5 mM EGTA, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 0.8 mM aprotinin, 21 μM leupeptin, 36 μM bestatin, 15 μM pepstatin A, 14 μM (4-guanidino) butane, and 1% Triton X-100. After centrifugation (12,000×, 10 min, 4 °C), the supernatants were used for Western immunoblot analysis. Appropriate volumes of the samples (10 μg/lane), were mixed with equal volumes of sample buffer (100 mM Tris–HCl, PH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.02% bromophenol blue), heated at 95 °C for 5 min, and then subjected to SDS–PAGE using 10% polyacrylamide gels. The proteins were transferred by semidry electroblotting from the gels to polyvinylidene difluoride membranes for 120 min. The blots were then blocked with the primary antibodies, cleaved caspase 9 or caspase 3 polyclonal antibody (Cell Signaling Technology, Denvers, MA, USA) for 18 h at 4 °C. Next, the blots were incubated with an appropriate second antibody, horseradish peroxidase-conjugated goat anti-rabbit IgG (Cell Signaling Technology), for 1 h. Immunoreactive bands