筑 波 大 学

博 士 （ 医 学 ） 学 位 論 文

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

（ゲンタマイシン耳毒性における蝸牛有毛細胞障害に対するスフィンゴシン１リン酸受容体アンタゴニストの効果）

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CONTENTS

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 [1,2,3,4]. 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 $S1PR_{1\text{-}5}$ [6,7,8,9]. 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 $S1PR_{1\text{-}3}$ 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.

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 (1μg) with a GeneAmp PCR System 9600 (Perkin Elmer, Tokyo, Japan). The mRNA expression levels of $S1PR_{1\text{-}5}$ 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 Eeagle's medium (DMEM) with 10% fetal bovine serum (FBS), 25mM HEPES, and 30U/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 hours) and then exposed to a medium containing 35μM gentamicin for 48 hours to assess the effects of S1PR antagonists

[18,19]. Each S1PR antagonist was tested at concentrations of 1 to100 μM.

2.4. S1P receptor antagonists

S1P and (R)-3-amino-(3-hexylphenylamino)-4- (oxobutylphosp-honic) 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-pyridin yl)-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, U.S.A.). 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 minutes and then permeabilized with 5% Triton X-100 (Sigma, St Louis, MO) in PBS with 10% fetal bovine serum (FBS) for 10 minutes. The specimens were stained with phalloidin with a conjugated Alexa Fluor probe (1:100, Molecular Probes, Carlsbad, CA) at room temperature for 1 hour. 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 to 20 explants per experimental group.

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, 21μM leupeptin, 36 μM bestatin, 15μM pepstatin A, 14μM (4-guanidino) butane, and 1% Triton X-100. After centrifugation (x12000, 10 minutes, 4°C), the supernatants were used for Western immunoblot analysis. Appropriate volumes of the samples (10μg/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 minutes, 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 minutes. 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 hours 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 hour. Immunoreactive bands were visualized using enhanced chemiluminescence(ECL kit; GE Healthcare Japan, Tokyo, Japan). The scanned immunoblot images were densitometrically analyzed with an ImageQuant LAS4000 mini imager (GE Healthcare Japan, Tokyo,

Japan). The result of each ratio of examined protein /β-actin protein was obtained from independent measurements (n=6 per group for each measurement).

2.8. Data analysis

All data were expressed as the means±SEMs. Statistical analysis was performed by unpaired *t* - tests or 1 way analysis of variance (ANOVA) followed by Bonferroni post-hoc tests, as required (StatView 5.0). Probability values less than 0.05 were considered significant.

3. Results

3.1 Expression of S1PRs in the cochlea

Nonquantitative RT-PCR analysis clearly detected $S1PR_2$ mRNA in the organ of Corti and spiral ganglion. $S1PR_1$ and $S1PR_3$ mRNA was also detected in both the organ of Corti and the spiral ganglion. In contrast to the results for $S1PR_{1-3}$, no expression of $S1PR_4$ or $S1PR_5$ mRNA was observed both in either the organ of Corti or the spiral ganglion (Fig.1).

3.2 Effects of S1PR antagonists on cochlear hair cells (control study)

In control explants maintained in the initial medium for 48 hours without exposure to gentamicin, almost all hair cells including 1 row of inner hair cells and 3 rows of outer hair cells were present (Fig.2A). The effects of each antagonist and each solvent (W146, DMSO; JTE013, Methanol; BML241,DMF) on the outer hair cells were examined and compared with the control explants. These antagonists and their solvents did not induce any significant outer hair cell loss when explants were cultured for 48 hours in the medium containing these agents (data not shown).

3.3 Effects of S1PR antagonists on gentamicin ototoxicity

The effect of each antagonist on gentamicin-induced cochlear hair cell loss was examined using basal turn explants. The explants treated with gentamicin alone showed significantly reduced numbers of outer hair cells (Fig. 2A). Compared to the explants treated with gentamicin alone (the gentamicin control group), JTE013 increased the ratio of outer hair cell loss induced by gentamicin at the concentrations of 10 and 100 μM (Fig. 2A and B) (Bonferroni test: $p < 0.05$). The ratio of outer hair cell loss differed significantly between the 10 and 100μM subgroups, suggesting the dose -dependency of outer hair cell damage within this range of concentrations (Bonferroni test: $p < 0.05$). However, W146 (1, 10,100μM) or BML241 (1, 10,100μM) did not influence gentamicin ototoxicity (Fig.2A and B).

3.4. Activation of the intrinsic apoptotic pathway in gentamicin ototoxicity

The protein expression levels of cleaved caspase-3 and cleaved caspase-9 in the cochlea were examined by Western blot analysis (Figs. 3 and 4). Neither cleaved caspase 3 nor cleaved caspase 9 was detected in the normal organ of Corti cultures (without gentamicin). Gentamicin induced expression of cleaved caspases 3 and 9. In addition, JTE013 treatment significantly

increased expression levels of cleaved caspases 3 and 9 as compared with the gentamicin-alone group.

4. Discussion

Expression of $S1PR_{1-3}$ in the organ of Corti was shown in previous studies [10,11]. In the present study, we demonstrated that $S1PR_{1-3}$ mRNA was expressed in the spiral ganglion as well as in the organ of Corti of Sprague-Dawley rats'. However, $S1PR_4$ and $S1PR_5$ mRNA was not detected in the cochlea. The present findings seem reasonable because $S1PR_{1-3}$ are widely expressed in various tissues, whereas expression of $S1PR_4$ is reportedly limited to the lymphoid and hematopoietic tissues and that of $S1PR_5$ to the central nervous system [10,20].

On the basis of the results obtained for S1PR expression in the cochlea, we examined the effects of $S1PR_{1-3}$ antagonists on gentamicin ototoxicity. The results showed that the ratio of outer hair cell loss increased in the JTE013-treated group as compared with in the gentamicin-alone group. On the other hand, W146 or BML241 treatment had no effect on the gentamicin-induced damage of hair cells. S1P, produced intracellularly, generally functions as an anti-apoptotic substance. In our previous study, S1P protected hair cells against gentamicin ototoxicity [5]. The present findings strongly suggest that $S1PR_2$ signaling is important for cochlear hair

cell survival in gentamicin ototoxicity. Kono et al. [10] and MacLennan et al. [21] recently reported that S1P signaling was essential for the maintenance of the cochlea in infantile animals via the activation $S1PR_2$. The $S1PR_2$ -null mice were profoundly deaf, and the structure of the cochlea in $S1PR_2$ null mice was abnormal [10,21]. Their findings support our assumption that $S1PR_2$ signaling influences the fate of cochlear hair cells during gentamicin exposure.

The mechanisms of aminoglycoside-induced cochlear hair cell death are not fully known. However, it has been reported that the intrinsic apoptotic pathway is one of the major pathways leading to cochlear hair cell death [15,22]. We examined the activation of the intrinsic apoptotic pathway by measuring cleaved caspases 3 and 9. In agreement with the previous reports [15,22], gentamicin activated the intrinsic apoptotic pathway. Namely, both cleaved caspases 3 and cleaved caspases 9 were detected after gentamicin exposure. Furthermore, JTE013, an $S1PR_2$ antagonist, enhanced the cleavage of caspases 3 and 9 induced by gentamicin. On the basis of the present finding, it is assumed that S1P may inhibit the intrinsic apoptotic pathway via $S1PR_2$ in gentamicin ototoxicity.

5. Conclusions

An $S1PR_2$ antagonist enhanced the intrinsic apoptotic pathway of cochlear outer hair cells in gentamicin ototoxicity. This finding suggests the importance of $S1PR_2$ signaling in cochlear hair cell survival against ototoxic insults.

6. Acknowledgments

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Figures

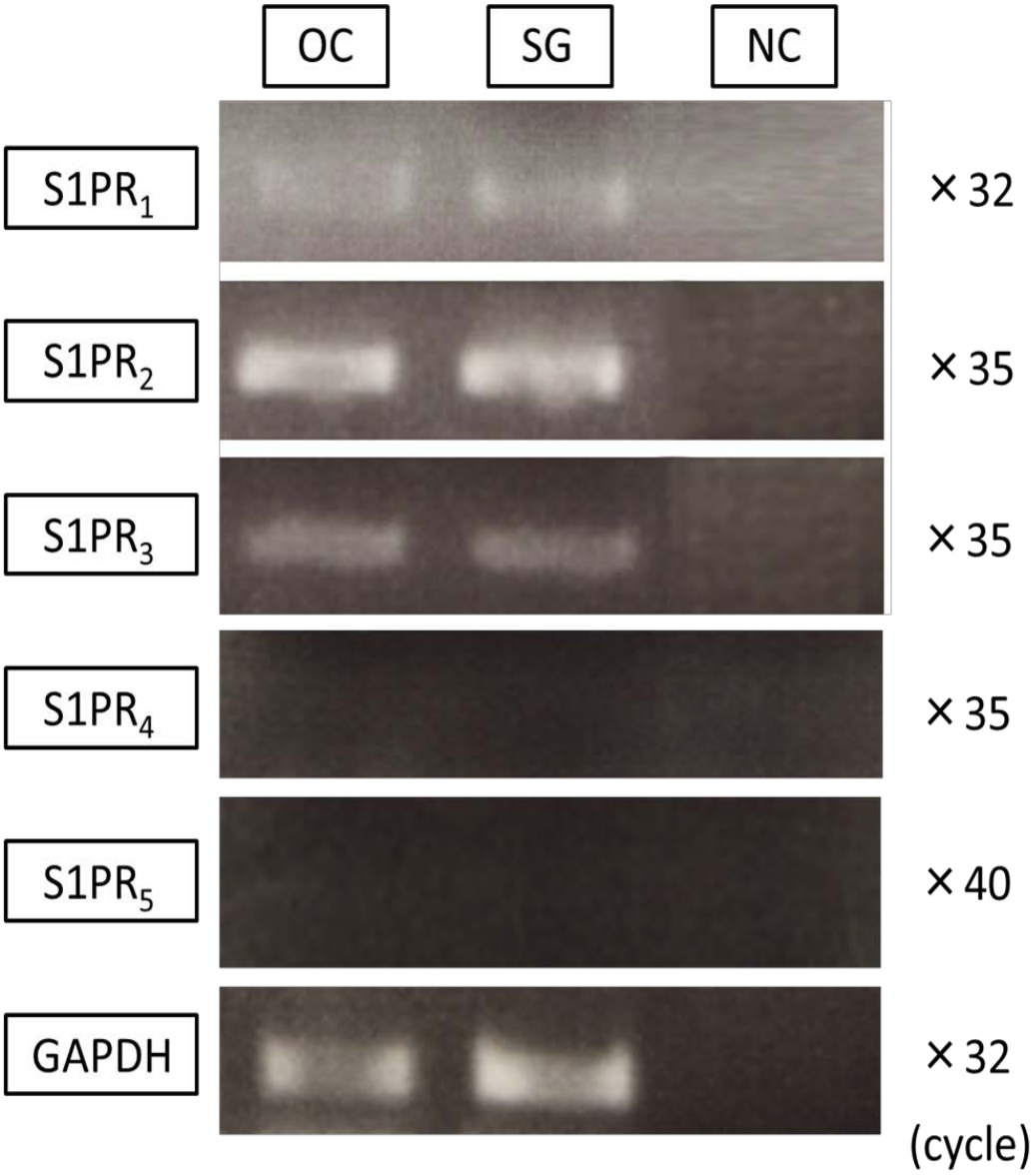
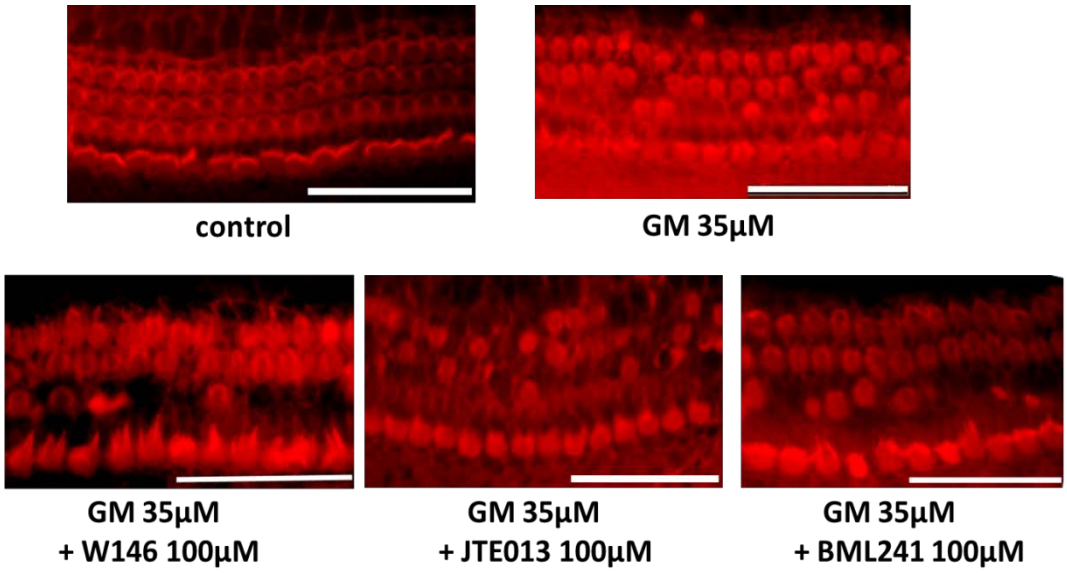


Figure 1



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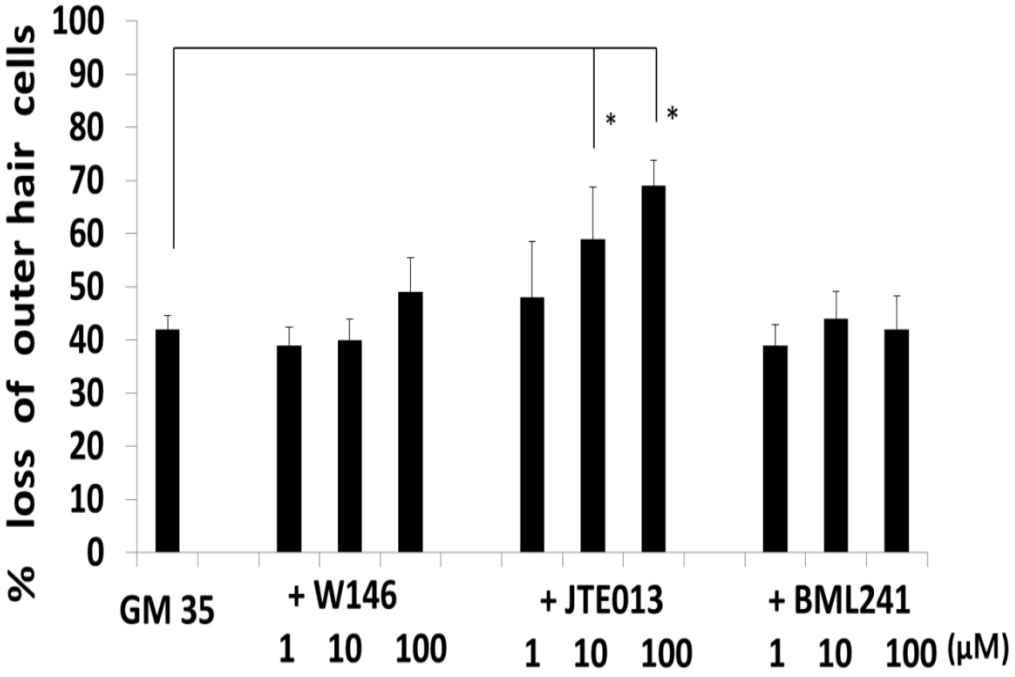

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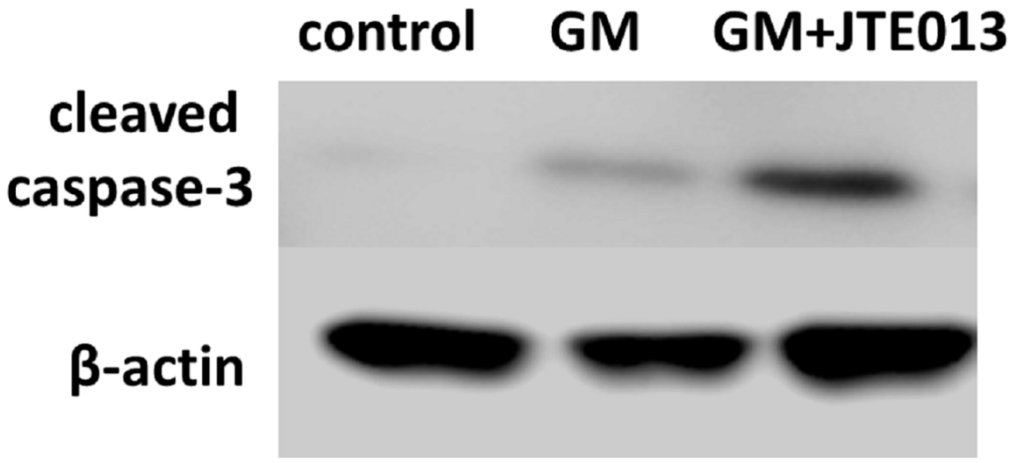
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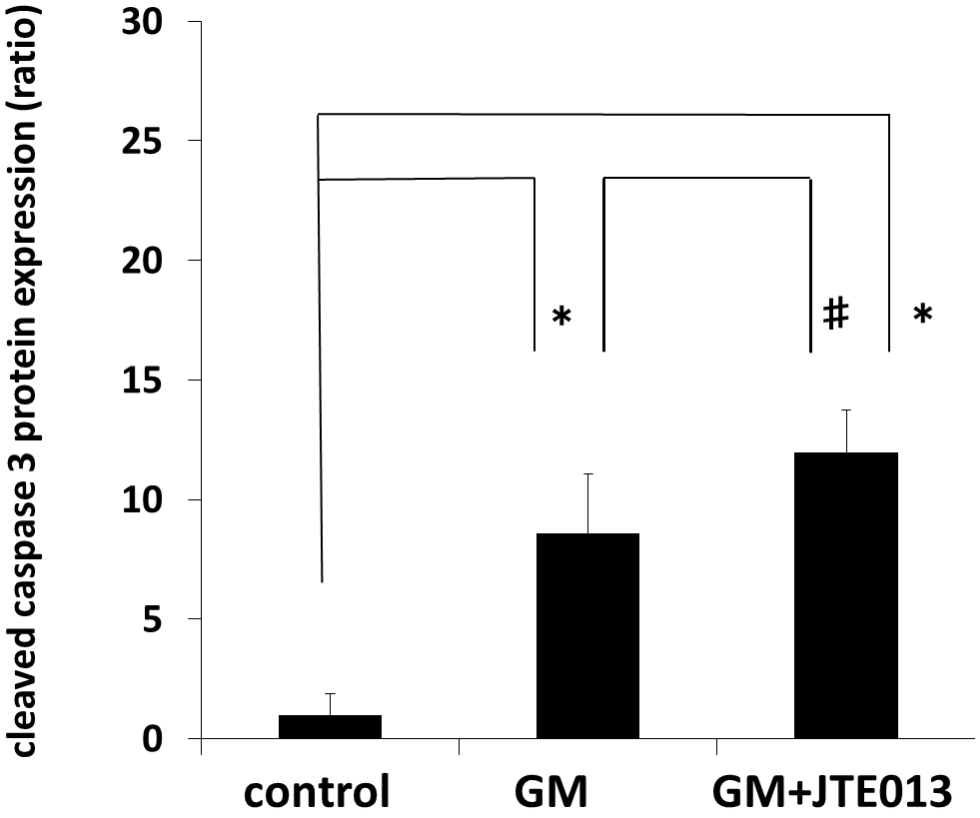
cleaved
caspase-3
β-actin

Figure 3(A)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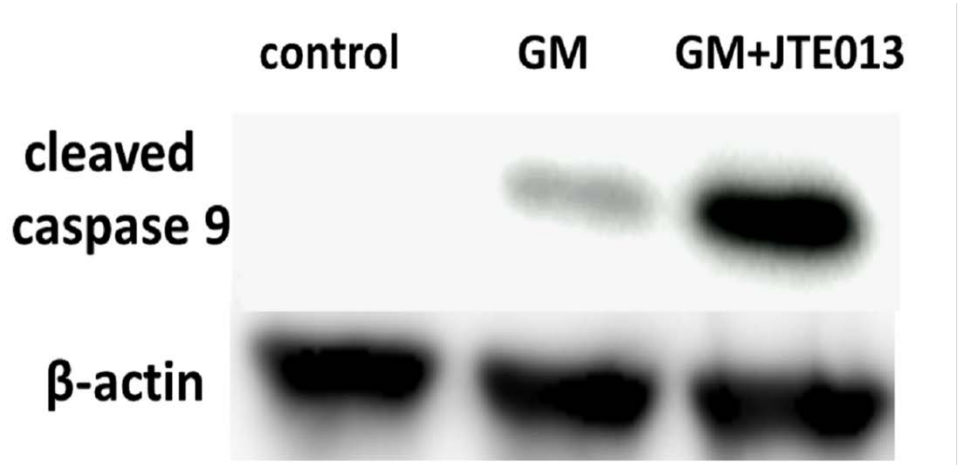
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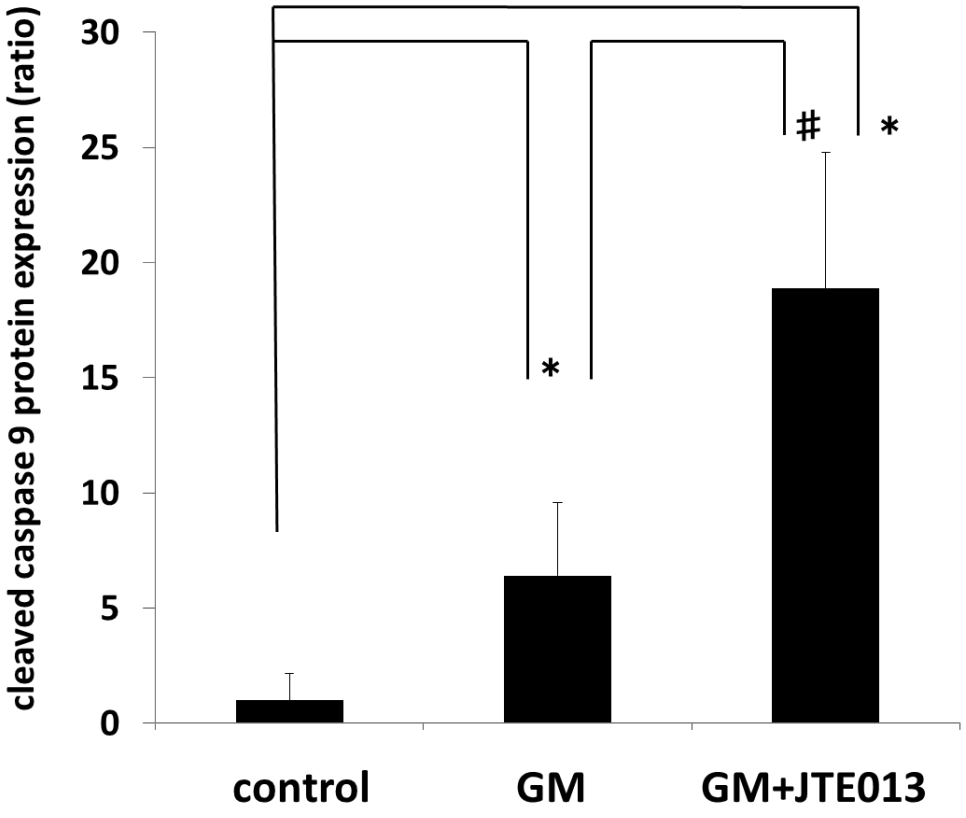
cleaved caspase 9 protein expression (ratio)
30
25
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5
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control
GM
GM+JTE013

Figure Legends

Figure 1. Expression of S1PRs in the cochlea. RT-PCR analysis demonstrated that 3 S1P receptors ($S1P_{1\text{-}3}$) 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)

Figure 2. Effects of $S1P_{1\text{-}3}$ antagonists on cochlear hair cells. The organ of Corti explant was cultured with or without gentamicin. (A) Representative microphotographs. (B) The effects of S1P antagonists on gentamicin ototoxicity were examined. The organ of Corti was cultured with 35 μM gentamicin alone or with 35μM gentamicin plus 100 μM of each S1PR antagonist for 48 hours. JTE013 (an $S1PR_2$ antagonist) increased hair cell loss at 10 and 100μM as compared with the culture with gentamicin alone ($p<0.05$, 1-way ANOVA and Bonferroni test). Scale bar: 50μm.

Figure 3. Expression levels of cleaved caspase 3 in the organ of Corti were assessed by Western blot analysis (n=6 in each group). (A) Typical blot

images. β-actin was used as an internal control. Cleaved caspase 3 was not detected in the control organ of Corti (without gentamicin). Cleaved caspase 3 was detected in the explant exposed to gentamicin. JTE013 increased expression levels of cleaved caspase 3. (B) Quantitative analysis of cleaved caspase-3. Control: tissue sample from organ of Corti maintained in the initial medium for 48 h. GM: organ of Corti treated with gentamicin 35 μM for 48 h. GM+JTE013: organ of Corti treated with 35 μM gentamicin plus 100 μM JTE013 for 48 h.

Figure 4. Expression levels of cleaved caspase 9 were assessed by Western blotting. (A) Typical blot images. β-actin was used as an internal control. Cleaved caspase 9 was not detected in the cotrol organ of Corti (without gentamicin). Gentamicin induced cleaved caspase 9, and JTE further increased expression level of cleaved caspase 9.
(B) Quantitative analysis of cleaved caspase-9. control; tissue sample from the organ of Corti maintained in the initial medium for 48h. GM; the organ of Corti treated with 35μM gentamicin for 48h. GM+JTE013; the organ of Corti treated with 35 μM gentamicin plus 100 μM JTE013 for 48 hours.