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博 士 （医 学） 学 位 論 文

Hair Cell Loss Induced by Sphingosine and a Sphingosine Kinase Inhibitor in the Rat Cochlea

（ラット蝸牛におけるスフィンゴシン及びスフィンゴシンキナーゼ阻害薬による内耳有毛細胞障害）

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筑波大学大学院博士課程人間総合科学研究科

谷　紘輔

Contents

Hair Cell Loss Induced by Sphingosine and a Sphingosine Kinase Inhibitor in the Rat Cochlea

Kohsuke Tani

Otology and Neuro-otology, Majors of Medical Sciences, Graduate School of Comprehensive Human Sciences,

University of Tsukuba, Tsukuba, Japan

Abstract

Sphingolipid metabolites including ceramide, sphingosine (Sph), and sphingosine-1-phosphate (S1P) play important roles in the regulation of cell survival and death. Sphingosine kinase (Sk) phosphorylates Sph to S1P. Sk is reportedly overexpressed in various cancer cells, and Sk inhibitors are therefore a target of anti-tumor therapy. However, the effects of Sph and Sk inhibitors on cochlear hair cells were unknown. In the present study, expression of Sk isotypes in the cochlea was examined. In addition, the changes in Sk activity induced by cisplatin (CDDP) and the effects of an Sk inhibitor, Sph and S1P on CDDP ototoxicity were investigated using tissue culture techniques. Cochleae were dissected from Sprague-Dawley rats on postnatal days 3 to 5. Organ of Corti explants were exposed to 5 μM CDDP for 48 h with or without the Sk inhibitor, Sph, or S1P. Both Sk1 and Sk2 were expressed in the normal cochlea. CDDP activated Sk. The Sk inhibitor itself caused hair cell loss at a high concentration, and at lower concentrations it increased CDDP-induced hair cell loss. Sph itself also induced hair cell death and increased hair cell loss induced by CDDP. However, S1P decreased hair cell loss induced by CDDP. Sk inhibitor has the function by increasing ototoxic Sph and decreasing otoprotective S1P, and therefore potentially cause ototoxicity. Consideration of the possibility of ototoxicity is required in the usage of Sk inhibitors.

Key words: Sphingosine; Sphingosine kinase; Cisplatin; Cochlea; Cochlear hair cell

Introduction

The sphingolipids are a class of complex lipids that contain organic aliphatic amino alcohols such as sphingosine (Sph) (Nikolova-Karakashian et al. 2011; Van Brocklyn et al. 2012). Sphingolipids not only act as a cell structure component but also play multiple roles in physiologic functions, such as apoptosis, cell growth arrest, differentiation, senescence, migration, and adhesion (Maceyka et al. 2012). Ceramide (Cer), composed of Sph and a fatty acid, is converted to Sph by ceramidase. Sph is, in turn, phosphorylated to sphingosine-1-phosphate (S1P) by sphingosine kinase (Sk) (Taha et al. 2006). Nishimura et al. (2010) reported that Cer increased cochlear hair cell loss induced by gentamicin, whereas S1P protected cochlear hair cells against gentamicin. On the basis of previous reports, balance between these two metabolites is thought to be important for determination of hair cell fate (Cuvillier et al. 2000, 2001; Maceyka et al. 2002; Ogretmen et al. 2004). However the effects of Sph on cochlear hair cells were remained unknown.

Sk is reportedly over expressed in many cancer cells (Xia et al. 2000; Nava et al. 2002; Guan et al. 2011; Liu et al. 2010, 2012; Pchejetski et al. 2011). Over-expressed Sk renders the malignant cells resistant to chemoradiotheraphy and thus results in poor patient prognosis (Olivera et al. 1999; Van Brocklyn et al. 2005; Bonhoure et al. 2006; Hait et al. 2006; Li et al. 2009; Liu et al. 2010). Pharmacologic Sk inhibition has been a target of for anti-cancer therapy (French et al. 2003, 2006; Huwiler et al. 2011; Pyne et al. 2011). In addition, combination of Sk inhibitors and diverse anti-tumor drugs including cisplatin (CDDP) has been attempted in various cancer cell lines to increase toxicity toward tumor cells (Kedderis et al. 1995; Min et al. 2004, 2005; Alexander et al. 2006; Dickson et al. 2011; Ishitsuka et al. 2014; Liu et al. 2014). CDDP is a

platinum-containing anti-cancer agent used for various malignant tumors (Rybak et al. 2007; Ding et al. 2011). However, CDDP may cause several adverse effects such as nephrotoxicity and ototoxicity (Rybak et al. 2007; Ding et al. 2011).

The effects of Sk inhibitors and Sph on cochlear hair cells are unknown. In addition, the effects of co-administration of Sk inhibitors and CDDP on hair cells have not been investigated. The present study therefore investigated (1) the expression of Sk in the rat cochlea, (2) the effect of CDDP on Sk activity in the cochlea, (3) the effect of an Sk inhibitor or Sph on cochlear hair cells, and (4) the effect of co-administration of an Sk inhibitor, Sph, or S1P and CDDP on cochlear hair cell death.

Materials and method

Animals

Postnatal day 3 to 5 Sprague-Dawley rats were used. For immunohistochemical evaluation of Sk1 and Sk2 in the mature cochlea, adult female Sprague-Dawley rats were used. All animal procedures were carried out according to the guidelines of the Laboratory Animal Research Center of the University of Tsukuba.

Sk expression in the cochlea

The organs of Corti and lateral walls were dissected according to the methods of Van de Water et al. (1974) and Sobkowicz et al. (1993). Briefly, after the decapitation, the ear capsule was quickly exposed and broken out, and the organ of Corti and the lateral wall were isolated. I used the whole turn of the organ of Corti and the lateral wall. Total RNA was extracted from each cell using Trizol (Invitrogen, Carlsbad, CA, USA).

Reverse transcription of the extracted total RNA was performed using random primer (Random Primers, Invitrogen), RNaseOUT™ Recombinant Ribonuclease Inhibitor (Invitrogen), and SuperScript® II Reverse Transcriptase (Invitrogen). The mRNA expression levels of Sk1 and Sk2 were detected by conventional Reverse transcription-polymerase chain reaction (RT-PCR) with Taq polymerase enzyme (Takara, Shiga, Japan) by GeneAmp PCR System 9600 (Perkin Elmer, Tokyo, Japan). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control for RNA integrity (Nakayama et al. 2014). Gene sequences from Sk1 (NM_001270811.1), Sk2 (NM_001012066.1), and GAPDH (NM_017008.4) were accessed from GenBank®. Primers for RT-PCR were designed using Primer-Blast software available at the NCBI (National Center for Biotechnology Information).

The primer sequences of Sk1 were as follows:

Sk1-F: 5`- CCCGGAGAGAACAAGGAACG -3` Sk1-R: 5`- CGGCCAGATCCTTTGAGGTT -3`

The predicted amplicon sizes are 573.

The primer sequences of Sk2 were as follows:

Sk2-F: 5`- AACCATCATGGCGGGTTTGA -3` Sk2-R: 5`- TGAAAGCGTTGGGGGATGAA -3`

The predicted amplicon sizes are 577.

The primer sequences of GAPDH were as follows:

GAPDH-F: 5`-AAGGTCATCCCAGAGCTGAA-3` GAPDH-R: 5`-GTTGAAGTCACAGGAGACAACC-3`

The predicted amplicon sizes are 207.

Western blot analysis was performed to detect protein expression levels of Sk1 and Sk2 at the organ

of Corti and lateral wall. The organs of Corti and lateral walls were dissected according to the methods of Van de Water et al. (1974) and Sobkowicz et al. (1993). Briefly, after the decapitation, the ear capsule was quickly exposed and broken out, and the organ of Corti and the lateral wall were isolated. I used the whole turn of the organ of Corti and the lateral wall. The organs of Corti and lateral walls were homogenized in PRO-PREP™ (Cosmo Bio, Tokyo, Japan). After centrifugation (13,000 rpm ×, 5 min, 4 ◦C), the supernatants were used for Western blot 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 % bromophenolblue), heated at 95 ◦C for 5 min, and then subjected to SDS–PAGE using 4-20 % Mini-PROTEAN® TGX™ Gel (Bio-Rad, Hercules, CA, USA**)**. The proteins were transferred by means of a semidry electroblotting system from the gels to polyvinylidene difluoride membranes for 1 h. The blots were then blocked with the primary antibodies for Sk1 (1: 500) (bs-2652R; Bioss Inc., Woburn, MA, USA) or Sk2 (1: 500) (bs-2653R; Bioss Inc.) for 18 h at 4 ◦C. Skim milk powder was used for blocking buffer. Next, the blots were incubated with an appropriate second antibody, horseradish peroxidase-conjugated goat anti-rabbit IgG (1: 2000) (7074S; Cell Signaling Technology, Denver, MA, USA), for 1 h. Immunoreactive bands were visualized using enhanced chemiluminescence (ECL kit; GE Healthcare Japan, Tokyo, Japan). Imaging was performed using ImageQuant LAS4000mini system (GE Healthcare Japan, Tokyo, Japan).

Immunofluorescence staining was performed to detect expression of Sk1 and Sk2 in the organ of Corti. The basal turns of the organ of Corti from postnatal day 3 to 5 were dissected. They were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min and then blocked in blocking buffer (PBS/5%

normal goat serum (Sigma, St Louis, MO, USA) /0.3% Triton™ X-100 (Sigma)) for 60 min. Primary antibody for Sk1 (1: 100) (bs-2652R; Bioss Inc.) or Sk2 (1: 100) (bs-2653R; Bioss Inc.) were applied overnight at 4°C. The specimens were then incubated in Anti-rabbit IgG (H+L), F(ab')2 Fragment (Alexa Fluor® 488 Conjugate) (1:100) (4412S; Cell Signaling Technology) with phalloidin (1:100) (Molecular Probes, Carlsbad, CA, USA) at room temperature in the dark for 1h. Phalloidin is a specific marker for cellular F-actin and labels the stereociliary arrays and the cuticular plates of hair cells (Tabuchi et al. 2007; Nishimura et al. 2010; Nakamagoe et al. 2010; Nakayama et al. 2014; Chi et al. 2015). Imaging was performed using a confocal laser scanning microscope (FLUOVIEW® FV10i, OLYMPUS, Tokyo, Japan).

For experiments on immunohistochemical localizations of Sk1 and Sk2 in the rat cochlea, adult female Sprague-Dawley rats (SLC, Hamamatsu, Japan; 220–250 g) were deeply anesthetized with pentobarbital sodium. The cochleae were quickly removed from the temporal bone, were fixed with 4% paraformaldehyde, decalcified in 10% EDTA at 4°C, and then embedded in paraffin. The cochleae were cut into 5 μm-thick sections and mounted on slides (Hoshino et al. 2011). For antigen retrieval, the de-paraffinized sections were boiled for 15 min in an autoclave in citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked through incubation with a 0.03% hydrogen peroxide solution for 20 min at room temperature. Each section was blocked in 5% goat serum (PK-6101; Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA, USA) for 30 min at room temperature. Primary antibody dilutions were prepared as follows: primary antibodies for Sk1 (1: 50) (bs-2652R; Bioss Inc.) or Sk2 (1: 50) (bs-2653R; Bioss Inc.) at 4°C overnight, followed by incubation with the biotinylated goat anti-rabbit antibody (PK-6101; Vectastain Elite ABC Kit, Vector Laboratories) at

37°C for 30 min. The signal was developed with SignalStain® DAB Substrate kit (8059; Cell Signaling Technology), and all slides were counterstained with hematoxylin. Negative controls were treated with PBS instead of the primary antibodies (data not shown) (Coleman B et al. 2009; Tanaka S et al. 2012; Tang Y et al. 2014). Imaging was performed using fluoroscence microscope BZ-X710 (KEYENCE, Osaka, Japan).

Culture technique

The basal turns of the organ of Corti were dissected and cultured. The cochlear explants were maintained in Dulbecco's modified Eagle's medium 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 % CO2 at 95 % humidity. The cochlear cultures were maintained in the above-described medium overnight (8-12 h) and then exposed to a medium containing 5 μM CDDP for 48 h to assess the effects of Sph, the Sk inhibitor and S1P (Tabuchi et al. 2007; Nishimura et al. 2010; Nakamagoe et al. 2010; Nakayama et al. 2014; Chi et al. 2015).

Chemicals

2-(p-hydroxyanilino)-4-(p-chlorophenyl)thiazole (SKi, an Sk inhibitor that inhibits both Sk1 and Sk2) and Sph were purchased from Echelon (Salt Lake City, UT, USA). S1P was purchased from Sigma. SKi, Sph and S1P were tested at 10-50 μM. SKi was initially dissolved in dimethyl sulfoxide (DMSO; Sigma) to 10 mM and stored at - 20 ◦C. Sph and S1P were initially dissolved in methanol (Wako Pure Chemical Industries, Osaka, Japan) to 10mM, and 2 mM, respectively, and stored at - 20 ◦C. Each agent was diluted in the culture medium to the final concentration immediately before use. The final concentrations of DMSO were 14-70 mM in SKi.

Cytochemistry

After the explants had been cultured for 48 h in culture media containing 5 μM CDDP alone or 5 μM CDDP plus each concentration of SKi or Sph or S1P, they were fixed with 4 % paraformaldehyde PBS for 20 min and then permeabilized with 5 % Triton™ X-100 (Sigma) in PBS with 10 % FBS for 10 min. The specimens were stained with phalloidin with a conjugated Alexa Fluorprobe (1:100; Molecular Probes) at room temperature for 1 h.

Assessment of cochlear hair cell damage

Imaging was performed using a confocal laser scanning microscope (FLUOVIEW® FV10i, OLYMPUS, Tokyo, Japan). The loss of hair cell was characterized as missing if no stereocilia and/or no cuticular plate were observed by phalloidin staining. I randomly assigned 3 parts from each culture. I counted the number of lost outer hair cells in 30 of outer hair cells in each part. The average of 3 parts was used to represent each culture (Tabuchi et al. 2007; Nishimura et al. 2010; Nakamagoe et al. 2010; Nakayama et al. 2014; Chi et al. 2015). The average of three separate counts was used to represent each culture.

Activation of caspase-3

The protein expression levels of caspase-3 at the organs of Corti were detected by conventional Western blot. The organs of Corti were homogenized in PRO-PREP™ (Cosmo Bio, Tokyo, Japan). After centrifugation (13,000 rpm ×, 5 min, 4 ◦C), the supernatants were used for Western blot 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 % bromophenolblue), heated at 95 ◦C for 5 min, and then subjected to SDS–PAGE using 4-20 % Mini-PROTEAN® TGX™ Gel (Bio-Rad, Hercules, CA, USA**)**.

The proteins were transferred by means of a semidry electroblotting system from the gels to polyvinylidene difluoride membranes for 1 h. The blots were blocked with the primary antibodies for β-actin (1: 1000) (4967S; Cell Signaling) or cleaved caspase-3 (1: 500) (9661S; Cell Signaling) for 18 h at 4 ◦C. Skim milk powder was used for blocking buffer. Next, the blots were incubated with an appropriate second antibody, horseradish peroxidase-conjugated goat anti-rabbit IgG (1: 2000) (7074S; Cell Signaling Technology), for 1 h. Immunoreactive bands were visualized using enhanced chemiluminescence (ECL kit; GE Healthcare Japan, Tokyo, Japan). The scanned immunoblot images were densitometrically analyzed with an ImageQuant LAS4000mini system (GE Healthcare Japan, Tokyo, Japan). The result of the cleaved caspase-3 protein/ β-actin protein assay was obtained from independent measurements (Nakayama et al. 2014).

Sphingosine kinase assay

The activity of Sk was quantified by using a Sphingosine Kinase Activity Assay (K-3500; Echelon) as described in the manufacturer's protocol. Briefly, organ of Corti cells were harvested and exposed to a medium containing 5 μM CDDP for 48 h. After the cells had been homogenized, 30 μg total proteins (16-20 organs of Corti) were incubated in reaction buffer containing 100 μM Sph and 10 μM ATP for 1 h at 37 ◦C. A luminescence-attached ATP detector was then added to stop the kinase reaction. The kinase activity was measured using a CentroXS3 LB960 microplate luminometer (Berthold Technologies, Bad Wildbad, Germany) (Cho et al. 2011; Guan et al. 2011).

Data analysis

All data were expressed as the means ± S.E.Ms. Statistical analysis was performed using unpaired *t*

-tests or one-way analysis of variance (ANOVA) followed by Bonferroni post hoc tests, as required (StatView 5.0). Probability values of less than 0.05 were considered significant.

Results

Sk expression in the cochlea

Expression of Sk1 and Sk2 was examined using the RT-PCR technique, Western blot analysis, and immunohistochemistry. Sk1 and Sk2 mRNAs were clearly detected in the organ of Corti and the lateral wall by nonquantitative RT-PCR analysis. Sk1 and Sk2 proteins were also clearly detected in the organ of Corti and the lateral wall by Western blot analysis (Fig. 1A, B). Immunofluorescence of Sk in the organ of Corti revealed the presence of both Sk subtypes in the inner and outer hair cells (Fig. 2). In addition, immunohistochemical evaluation for adult rats demonstrated that both Sk subtypes were expressed in the mature cochlea (Fig. 3).

Sk activation by CDDP treatment

Changes in Sk activity induced by CDDP exposure were examined using organ of Corti explants. Compared with the non-CDDP exposed samples, 5 μM CDDP significantly increased Sk activity at 48 h (Fig. 4; unpaired *t* -test: $P < 0.05$ in the 5 μM subgroups [n=4] as compared with the control group [n=3]).

Effects of SKi on cochlear hair cells

In control explants maintained in the initial medium for 48 h without exposure to CDDP, almost all hair cells including 1 row of inner hair cells and 3 rows of outer hair cells were present (Fig. 5A). The effects of SKi and DMSO as solvents on the outer hair cells were examined and compared with the control explants.

DMSO alone did not induce hair cell loss (data not shown). No significant hair cell loss occurred when the explants were cultured for 48 h in the medium containing 10 (n = 15) or 20 (n = 14) μM SKi alone (Fig. 5B). However, 50 μM SKi (n = 15) significantly induced hair cell loss as compared with the control group (n =8) (one-way ANOVA and Bonferroni test: $P < 0.05$ in the 50 μM subgroups).

Effects of SKi on CDDP ototoxicity

The effect of SKi on CDDP-induced cochlear hair cell loss was examined using organ of Corti explants. The explants treated with 5 μM CDDP alone showed reduced numbers of outer hair cells (Fig. 6A). When compared with the explants treated with CDDP alone (the CDDP control group, n = 22), SKi significantly and dose-dependently increased the ratio of outer hair cell loss induced by CDDP at the concentrations of 10 (n = 9), 20 (n = 10), and 50 (n = 10) μM (Fig. 6B; one-way ANOVA and Bonferroni test: $P < 0.05$ in the 10, 20, and 50 subgroups).

Activation of caspase-3 under SKi ototoxicity

The protein expression levels of cleaved caspase-3 in the cochlea were examined by Western blot analysis. Cleaved caspase-3 was expressed in the cochlea treated with 20 μM SKi alone, 5 μM CDDP alone, and 20 μM SKi plus 5 μM CDDP within 48 h (Fig. 7A). In addition, the group treated with 20 μM SKi plus 5 μM CDDP expressed a significantly higher level of cleaved caspase-3 than did the groups treated with 20 μM SKi alone or 5 μM CDDP alone at 48 h (Fig. 7B; one-way ANOVA and Bonferroni test: $P < 0.05$, n = 6 in each group).

Effects of Sph on cochlear hair cells

The effects of Sph and methanol as solvents on outer hair cells were examined. No significant hair cell loss occurred when explants were cultured for 48 h in the medium containing methanol alone (data not shown) and 10 (n = 12) or 25 (n = 18) μM Sph (Fig. 8A). However, 50 μM Sph (n = 19) significantly induced hair cell loss as compared with the control group (n = 12) (Fig. 8B; one-way ANOVA and Bonferroni test: $P < 0.05$ in the 50 μM subgroup).

Effects of Sph on CDDP ototoxicity

The effect of Sph on CDDP-induced cochlear hair cell loss was examined using organ of Corti explants (Fig. 9A, B). When compared with the explants treated with CDDP alone (n = 22), Sph increased the ratio of outer hair cell loss induced by CDDP at the concentrations of 10 (n = 11), 25 (n = 11), and 50 (n = 14) μM in a dose-dependent manner at 48 h (one-way ANOVA and Bonferroni test:$P < 0.05$).

Activation of caspase-3 under Sph ototoxicity

Although cleaved caspase-3 was not detected in the organ of Corti explants without Sph, cleaved caspase-3 was expressed in cochlea treated with 25 or 50 μM Sph alone (Fig. 10A). In addition, the group treated with 50 μM Sph alone (n = 6) showed significantly increased expression levels of cleaved caspase-3 as compared with the 25 μM Sph alone group (n = 5) and the group without Sph (n = 5) (Fig. 10B; one-way ANOVA and Bonferroni test: $P < 0.05$).

Effects of S1P on CDDP ototoxicity

The effect of S1P on CDDP-induced cochlear hair cell loss was examined using organ of Corti explants (Fig. 11A, B). When compared with the explants treated with CDDP alone (n = 22), S1P did not exhibit

any significant effect on CDDP-induced hair cell loss at 10 (n = 10) or 20 (n = 10) μM at 48h. However, S1P decreased the ratio of outer hair cell loss at 50 (n = 12) μM (one-way ANOVA and Bonferroni test:$P < 0.05$).

Discussion

Sk is a key enzyme in the pathway of phospholipid catabolism and controls the levels of two bioactive sphingolipids, Sph and S1P (Fig. 12). Two Sk isoforms, Sk1 and Sk2, are known (Pitson. 2011; Maceyka et al. 2012). These isoforms are located on separate genes but are highly homologous, with a similarity percentage of approximately 80%. Molecular weight of Sk1 is approximately 43 kDa and Sk2 is approximately 65 kDa (Wattenberg et al.2006). Unlike Sk1, Sk2 possesses an extended N-terminal tail (Liu et al. 2000; Alemany et al. 2007; Hait et al. 2009). The present PCR study and Western blot analysis demonstrated that mRNAs and proteins of both Sk subtypes were expressed in the cochlea (Fig. 1A, B). The immunofluorescence staining for postnatal day 3 to 5 rats and immunohistochemical evaluation for adult rats demonstrated that both Sk subtypes were expressed in the cochlea (Fig. 2, Fig. 3). Some functional redundancy was thought to exist between Sk1 and Sk2 because major phenotypes were not observed in either Sk1 or Sk2 single-knockout mice (Mizugishi et al. 2005). In in vitro studies, it has been reported that Sk1 causes cell proliferation, whereas Sk2 plays a role in both cell growth and apoptosis (Igarashi et al. 2003; Liu et al. 2003; Okada et al. 2005; Olivera et al. 1999; Pitman et al. 2010; Pitson. 2011). However, the functional differences between these subtypes were not fully understood.

SKi, used in this study, inhibits both Sk1 and Sk2 (Pitson 2011; Pyne et al. 2011). SKi itself induced cochlear hair cell death at a high concentration of 50 μM (Fig. 5A, B). In addition, when SKi was

co-administrated with CDDP, it reinforced the ototoxicity of CDDP even at the low concentrations at which SKi itself did not show any ototoxicity (Fig. 6A, B). The mechanisms of Sk inhibitor-related cell death are not fully known. However, it has been reported that the apoptotic pathway is one of the mechanism leading to cell death by Sk inhibitors (Cuvillier et al. 2000, 2001; Hait et al. 2006; Phillips et al. 2007; Kanno et al. 2011; Young et al. 2013). I examined the apoptotic pathway of the organ of Corti by detecting cleaved caspase-3. SKi induced the cleavage of caspase-3 in the organ of Corti. Furthermore, SKi accelerated the CDDP-induced apoptotic death of hair cells (Fig. 7A, B). On the basis of these findings, I can assume that cochlear hair cell death induced by SKi is related to apoptosis.

The exogenous administration of Sph showed ototoxicity when administered at a high concentration, but not at a low concentration (Fig. 8 A, B). Similar to SKi, when Sph was co-administered with CDDP, even at a low concentration, it augmented the ototoxicity of CDDP (Fig. 9A, B). Western blot analysis also showed that the apoptotic pathway was involved in hair cell death induced by Sph (Fig. 10A, B). Sph has been found to break plasma membrane permeability barrier (Contreras et al. 2006; Jiménez-Rojo et al. 2014). Also phytosphingosine, a member of the Sph family causes a direct permeabilization of the plasma membrane of yeast (Veerman et al. 2010). It is assumed that exogenous administration of Sph caused a permeabilization of the plasma membrane to the outer hair cells and an influx of Sph which resulted to apoptosis. On the other hand, S1P protected hair cells against CDDP (Fig. 11A, B). This result is in accordance with the protective effects of S1P on gentamicin ototoxicity (Nishimura et al. 2010). S1P exerts its cellular responses through a family of 5 G-protein-coupled S1P receptors (S1PRs) known as S1PR1-5 (Nakayama et al. 2014). S1PR1-3 are expressed in

the spiral ganglion and in the organ of Corti (Herr et al. 2007; Kono et al. 2007; Nakayama et al. 2014). Especially S1PR2 is detected in the supporting cells and in the inner and outer sensory hair cells (Herr et al. 2007). S1P exerts its cochlear protection against gentamicin ototoxicity via activation of S1PR2 (Nakayama et al. 2014). From those previous reports exogenous administration of S1P might protect hair cells against CDDP ototoxicity via activation of S1PR2 expressed in hair cells.

Cer, Sph and S1P are thought to be important for determination of hair cell fate (Cuvillier et al. 2000, 2001; Maceyka et al. 2002; Ogretmen et al. 2004). S1P causes cell survival, while Cer and Sph cause cell death (Fig. 12) (Spiegel et al. 1999; Pyne et al. 2002). Sph has been reported as promoter of apoptosis (Nikolova-Karakashian et al. 2000). Cytochrome c release from mitochondria and activation of caspases-8 and caspase-9 were observed with Sph in Jurkat cells (Cuvillier et al. 2000). Sph-mediated apoptosis requires Bax, pro-apoptotic member of the Bcl-2 protein family. Sph-mediated apoptosis requires translocation of Bax to the mitochondria and its activation that induces the release of cytochrome c and Smac/Diablo from the mitochondria in rhabdomyosarcoma cell line (Phillips et al .2007). Sph-induced apoptosis is also observed in hippocampal neurons and astrocytes (Kanno et al. 2011). On the other hand S1P has been considered as a counterbalance (Payne et al. 2002; Oskouian et al. 2010). In Jurkat cells, caspase-3 and caspase-7 activation by the translocation of cytochrome c and Smac/Diablo from mitochondria could be prevented by S1P (Cuvillier et al. 1998; Cuvillier et al. 2001). S1P is produced by phosphorylation of Sph due to Sk. Sk therefore regulates the effects of pro-apoptotic Sph and pro-proliferative S1P (Fig. 12). Enforced expression of Sk can protect cancer cells from apoptosis (Cuvillier et al. 2008). Inhibiting Sk and downregulating S1P is thus a rational therapeutic target in

cancer (Reynolds et al. 2004; Min et al. 2004, 2005; Cuvillier et al. 2008; Dickson et al. 2011; Pyne et al. 2011). From the view-point of cancer treatment, modulating the levels of sphingolipids like Sk inhibition is expected to augment the effect of existing anti-cancer drugs such as CDDP (Min et al. 2004, 2005; Alexander et al. 2006; Dickson et al. 2011; Ishitsuka et al. 2014; Liu et al. 2014). In D. discoideum, overexpressing Sk or null for S1P lyase, the enzyme that degrades S1P, have decreased sensitivity to CDDP, whereas cells null for the Sk or overexpressing S1P lyase are more sensitive (Min et al. 2004, 2005; Alexander et al. 2006). Some researchers suggested that inhibition of the sphingosine kinase synergistically increased sensitivity to CDDP and induced cell death (Min et al. 2004, 2005; Ishitsuka et al. 2014; Liu et al. 2014). Several survival pathways including, the extracellular signal-regulated kinase mitogen-activated protein kinase pathway, the phosphatidylinositol 3-kinase/Akt/mTOR pathway and the c-Jun N-terminal kinase pathway suggested to play key roles in synergy of Sk inhibitor and CDDP (Min et al. 2005; Ishitsuka et al. 2014; Liu et al. 2014). Also this synergy is suggested that Sk inhibitor increased the concentrations of CDDP in cell by downregulation of drug transport proteins (Liu et al. 2014). I showed for the first time the phenomenon that Sk inhibitor has an ototoxic effect by its pro-apoptotic nature which will also enhances the CDDP-induced apoptotic death results in ototoxicity (Fig. 7 A, B). We should be aware of co-administration of Sk inhibitor and an ototoxic drug in clinical usage might increase the possibility of ototoxicity. Although in my study I could neither proved the direct interaction of sphingolipids with CDDP nor the changes of sphingolipid in the cell. Further research will be necessary to reveal the exact protective and degenerative mechanisms of sphingolipids in hair cell.

CDDP activated Sk in the organ of Corti (Fig. 4). Activation of Sk seems reasonable for the protection

of the organ of Corti against CDDP because Sk functions by increasing S1P and decreasing Sph. Sk activity and expression are also stimulated by hypoxia (Ader et al. 2009; Ahmad et al. 2006). Hypoxia generates S1P as a result of increased activity of Sk and enhance the proliferation of smooth muscle cells (Yun et al. 2002). Hypoxia increases Sk activity via reactive oxygen species (ROS) which are apoptotic (Ader et al. 2009). This is same phenomenon shown in this study. To protect from cytotoxic agent such as CDDP, it is assumed that cell activate Sk for producing protective S1P. CDDP induces ROS in hair cells and consequently causes apoptosis (Rybak et al. 2007). ROS is also induced by several sphingosine kinase inhibitors including SKi (Chatzakos et al. 2012; Hagihara et al. 2014; Hamada et al. 2014). It is presumed that Sk inhibitor enhance CDDP ototoxicty synergistically by increasing more ROS than CDDP alone and impeding the intrinsic cell protective mechanism of the cochlea against CDDP ototoxicity.

In conclusion, SKi and Sph showed ototoxicity by themselves and enhanced the apoptotic death of outer hair cells in the presence of CDDP. Conversely S1P showed otoprotective effect on CDDP ototoxicity. From the view-point of growing evidence that Sk inhibitors are effective in the treatment of cancer and inflammatory diseases, more attention should be paid to the possibility of their adverse effects, including ototoxicity, before consideration of their clinical usage.

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Figure Captions

Fig. 1 Sk expression in the cochlea. (A) RT-PCR analysis demonstrated that Sk1 and Sk2 were expressed in the organ of Corti (OC) and lateral wall (LW). GAPDH primers served as the cDNA loading control. (B) Western blot analysis demonstrated that Sk1 and Sk2 were expressed in OC and LW. M: marker; NC: negative control; L: liver (positive control).

Fig. 2 Sk expression in the pup cochlea. Immunofluorescence staining demonstrated that Sk1 and Sk2 were expressed in the sterocilia of hair cells and cellular membrane. The outer and inner hair cells stained with Sk1. (A) Sk1 (indicated in green) (B) phalloidin (indicated in red) (C) Merged images of Sk1 and phalloidin. Sk1 is expressed in cytoplasm. (D) Sk1 (indicated in green) (E) phalloidin (indicated in red) and DAPI (indicated in blue) (F) Merged images of Sk1, phalloidin and DAPI. The outer and inner hair cells stained with Sk2. (G) Sk2 (indicated in green) (H) phalloidin (indicated in red) (I) Merged images of Sk2 and phalloidin. Sk1 is expressed in cytoplasm. (J) Sk2 (indicated in green) (K) phalloidin (indicated in red) and DAPI (indicated in blue) (L) Merged images of Sk2, phalloidin and DAPI. Scale bar: 40 μm. O: outer hair cell. I: inner hair cell

Fig. 3 Sk expression in the mature cochlea. Immunofluorescence staining demonstrated that Sk1 ((A) X400) and Sk2 ((B) X400) were expressed in the organ of Corti. Scale bar: 100 μm.

Fig. 4 Sk activation by CDDP. 48 h CDDP treatment activated Sk in the organ of Corti (unpaired *t* -test: **P* <

0.05 in the 5 μM subgroups).

Fig. 5 Effects of SKi on cochlear hair cells. Organs of Corti were cultured with 0 (control) to 50 μM SKi for 48 h. (A) Representative microphotographs of cochlear hair cells. Scale bar: 40 μm. (B) Quantitative analysis of outer hair cell loss induced by SKi. SKi increased hair cell loss at 50 μM as compared with the control group (one-way ANOVA and Bonferroni test: $*P < 0.05$).

Fig. 6 Effects of SKi on CDDP ototoxicity. Organs of Corti were cultured with 5 μM CDDP alone or with 5 μM CDDP plus 10-50 μM SKi for 48 h. (A) Representative microphotographs. Scale bar: 40 μm. (B) Quantitative analysis of outer hair cell loss. Each concentration of SKi increased hair cell loss as compared with CDDP alone (one-way ANOVA and Bonferroni test: $*P < 0.05$).

Fig. 7 Cleaved caspase-3 expression under SKi ototoxicity. (A) Representative blot images. β-Actin was used as an the internal control. Although cleaved caspase-3 was not detected at 0 h, expression of cleaved caspase-3 was clearly detected within 48 h in the 20 μM SKi alone, 5 μM CDDP alone, and 20 μM SKi plus 5 μM CDDP groups. (B) Quantitative analysis of cleaved caspase-3 expression. 20 μM SKi plus 5 μM CDDP increased cochlear hair cell loss as compared with 20 μM SKi alone and 5 μM CDDP alone at 48 h (one-way ANOVA and Bonferroni test: $*P < 0.05$).

Fig. 8 Effects of Sph on cochlear hair cells. Organ of Corti explants were cultured with or without Sph. (A) Representative microphotographs. Scale bar: 40 μm. (B) Quantitative analysis of outer hair cell loss induced by Sph. Organs of Corti were cultured with 10-50 μM Sph for 48 h. Sph increased hair cell loss at 50 μM (one-way ANOVA and Bonferroni test: $*P < 0.05$).

Fig. 9 Effects of Sph on CDDP ototoxicity. Organs of Corti were cultured with 5μM CDDP alone or with 5 μM CDDP plus 10-50 μM Sph for 48 h. (A) Representative microphotographs. Scale bar: 40 μm. (B) Quantitative analysis of outer hair cell loss. Each concentration of Sph increased hair cell loss as compared with the culture with CDDP alone (one-way ANOVA and Bonferroni test: $*P < 0.05$).

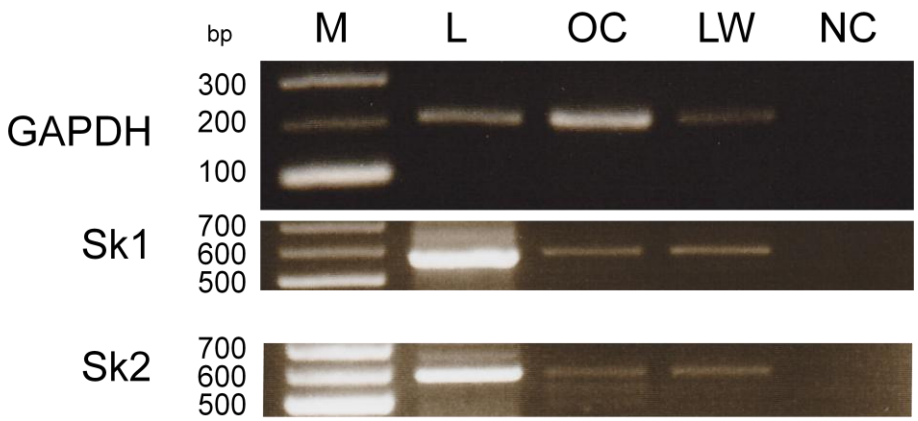
Fig. 10 Cleaved caspase-3 expression under Sph ototoxicity. (A) Representative blot images. β-actin was used as the internal control. Cleaved caspase-3 was not detected in the control organs of Corti (without Sph). Cleaved caspase-3 was detected in the explants exposed to Sph for 48 h. (B) Quantitative analysis of cleaved caspase-3. Cleaved caspase-3 was expressed at significantly higher levels in the 50 μM Sph group at 48 h than in the 25 μM Sph group and the group without Sph(one-way ANOVA and Bonferroni test: $*P < 0.05$).

Fig. 11 Effects of S1P on CDDP ototoxicity. Organs of Corti were cultured with 5 μM CDDP plus 10-50 μM S1P for 48 h. (A) Representative microphotographs of hair cells. Scale bar: 40 μm. (B) Quantitative analysis of outer hair cell loss. S1P decreased hair cell loss at 50 μM compared with the culture with CDDP alone (one-way

ANOVA and Bonferroni test: *$P < 0.05$).

Fig. 12 A schematic panel of roles of ceramide, sphingosine, sphingosine-1-phosphate, sphingosine kinase and SKi (sphingosine kinase inhibitor). I showed in my study that sphingosine and SKi has an apoptotic, cytotoxic effect for hair cells and sphingosine-1-phosphate has a protective effect against cisplatin.

(A)


bp
M
L
OC
LW
NC
GAPDH
300
200
100
Sk1
700
600
500
Sk2
700
600
500

(B)

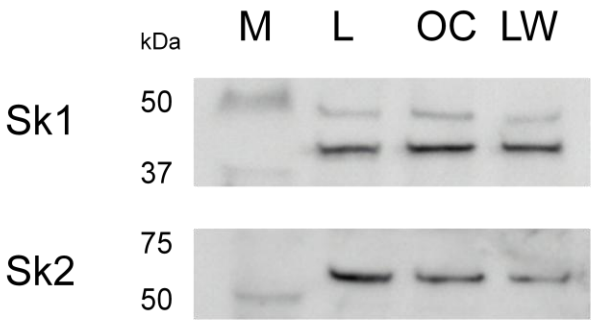

kDa
M
L
OC
LW
Sk1
50
37
Sk2
75
50

Fig. 1

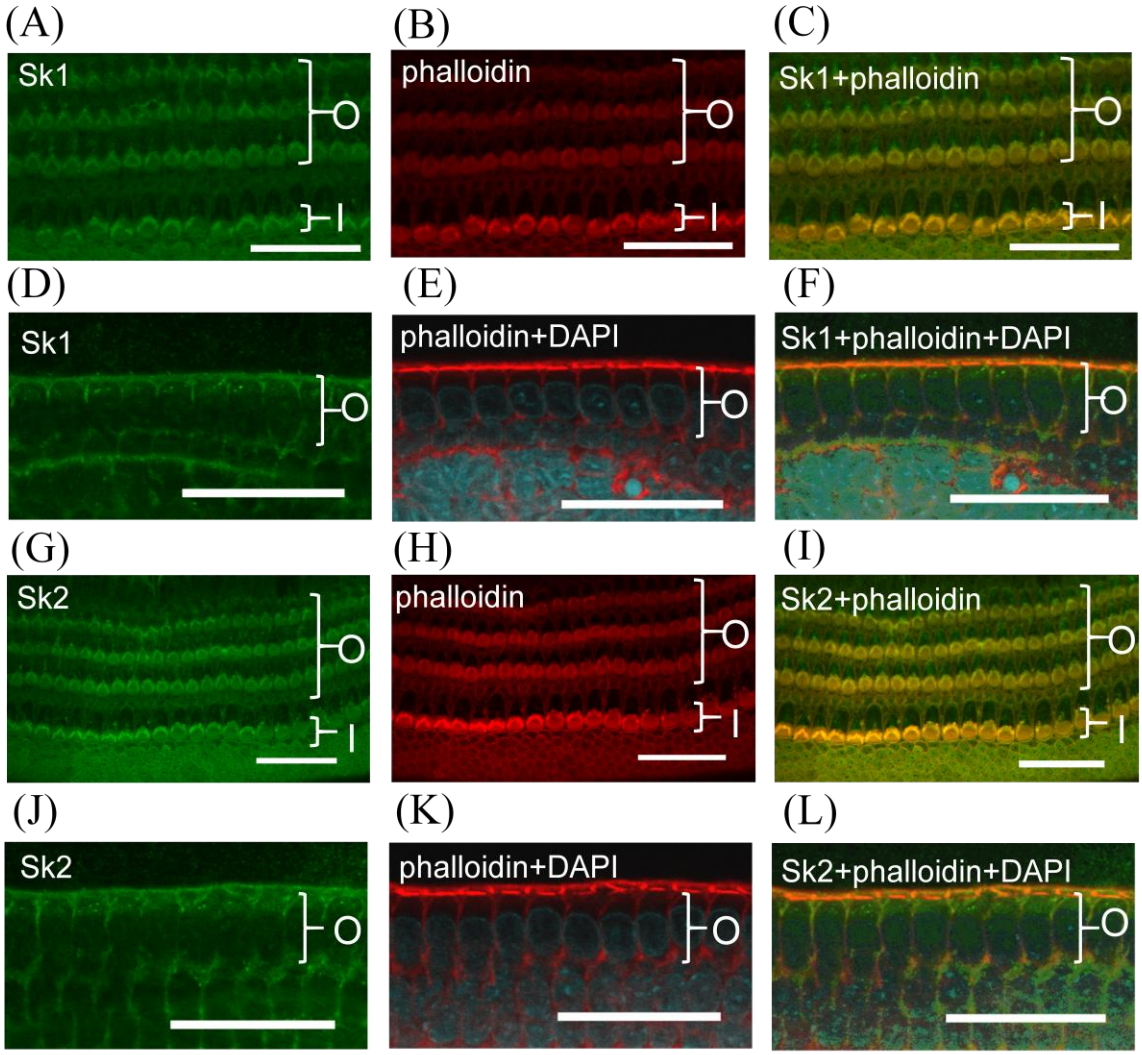

(A)
Sk1
(B)
phalloidin
(C)
Sk1+phalloidin
(D)
Sk1
(E)
phalloidin+DAPI
(F)
Sk1+phalloidin+DAPI
(G)
Sk2
(H)
phalloidin
(I)
Sk2+phalloidin
(J)
Sk2
(K)
phalloidin+DAPI
(L)
Sk2+phalloidin+DAPI

Fig. 2

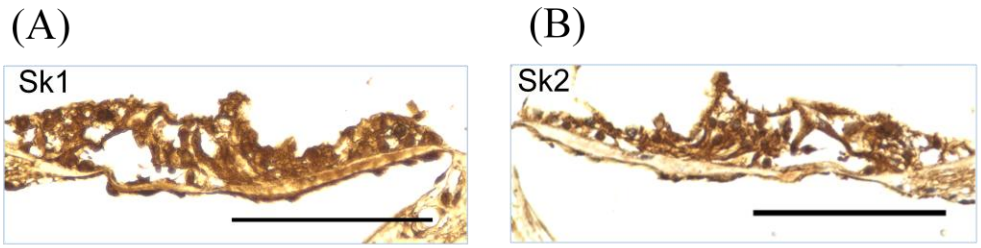

(A)
Sk1
(B)
Sk2

Fig. 3

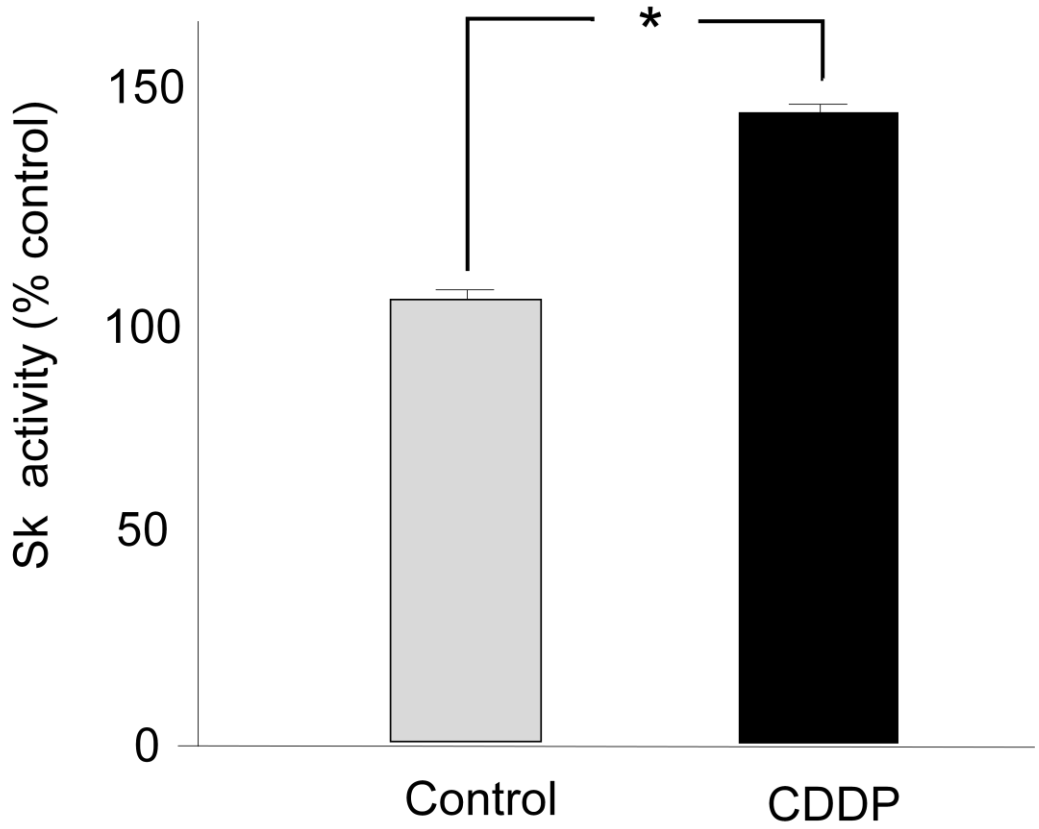


Fig. 4

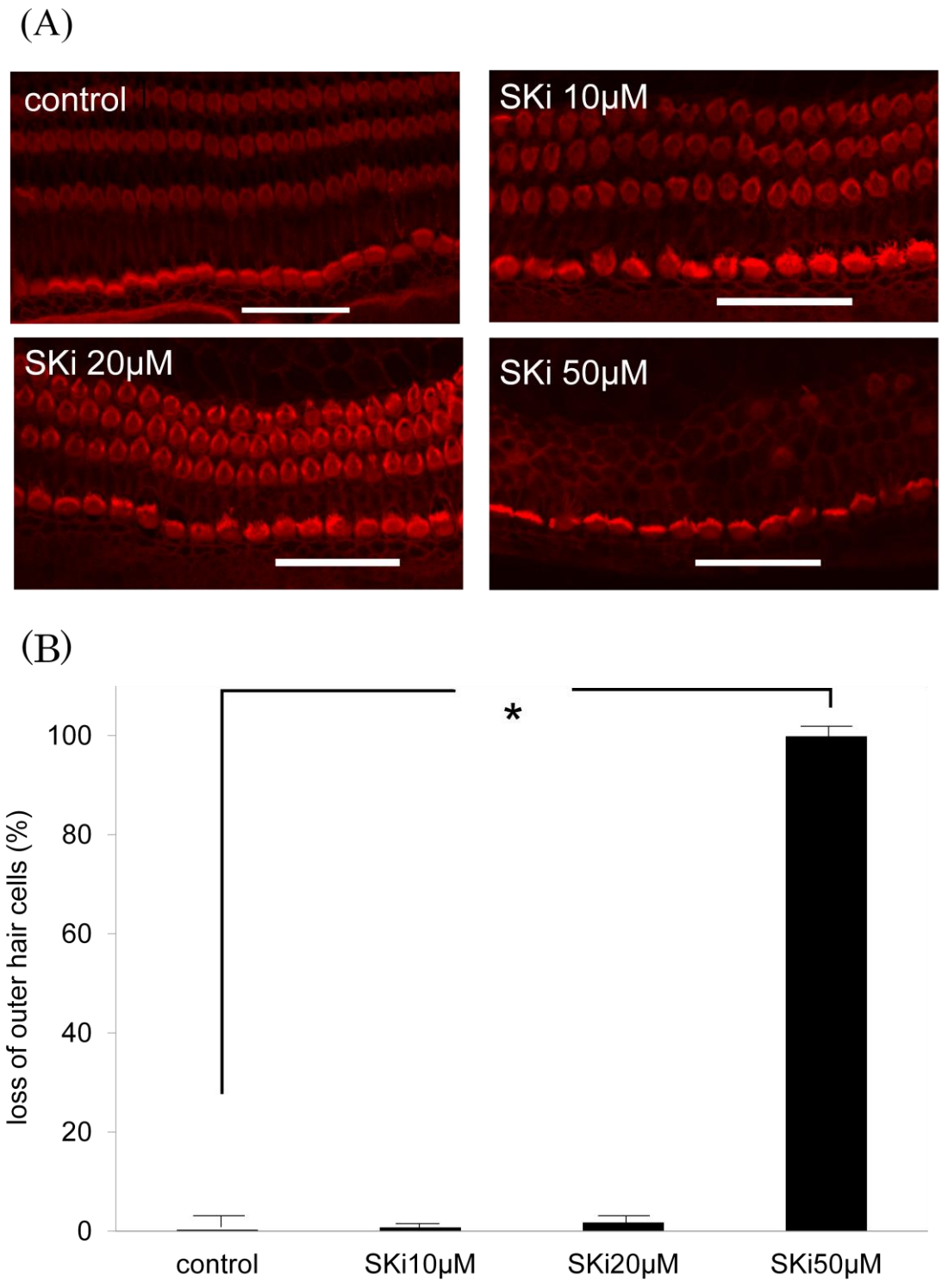
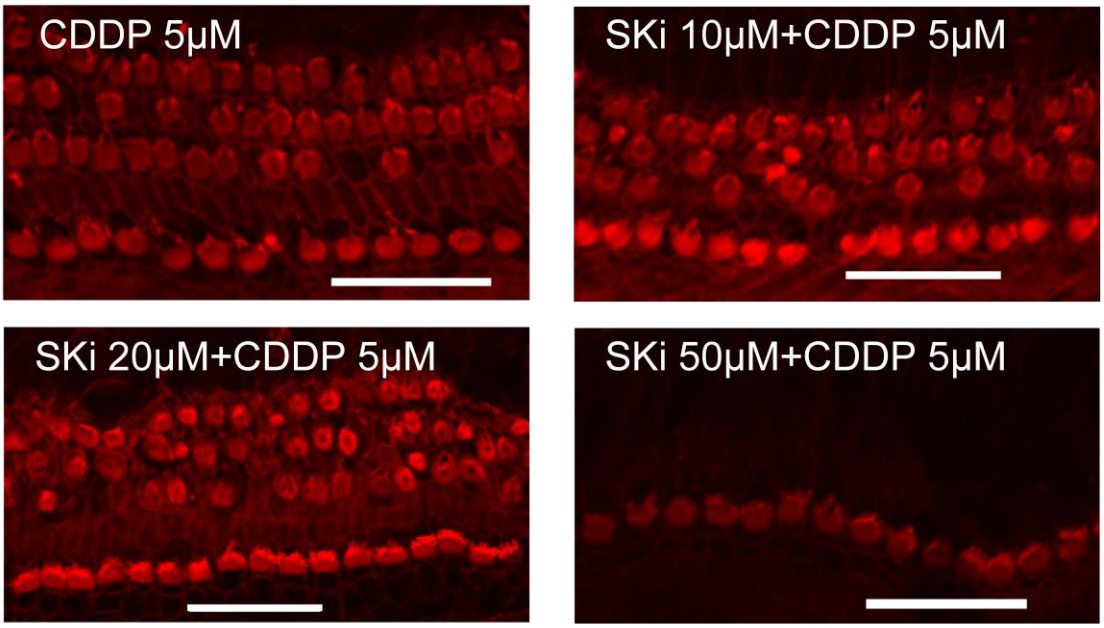

(A)
control
SKi 10μM
SKi 20μM
SKi 50μM
(B)
loss of outer hair cells (%)
100
80
60
40
20
0
*
control
SKi10μM
SKi20μM
SKi50μM

Fig. 5

(A)


CDDP 5μM
SKi 10μM+CDDP 5μM
SKi 20μM+CDDP 5μM
SKi 50μM+CDDP 5μM

(B)

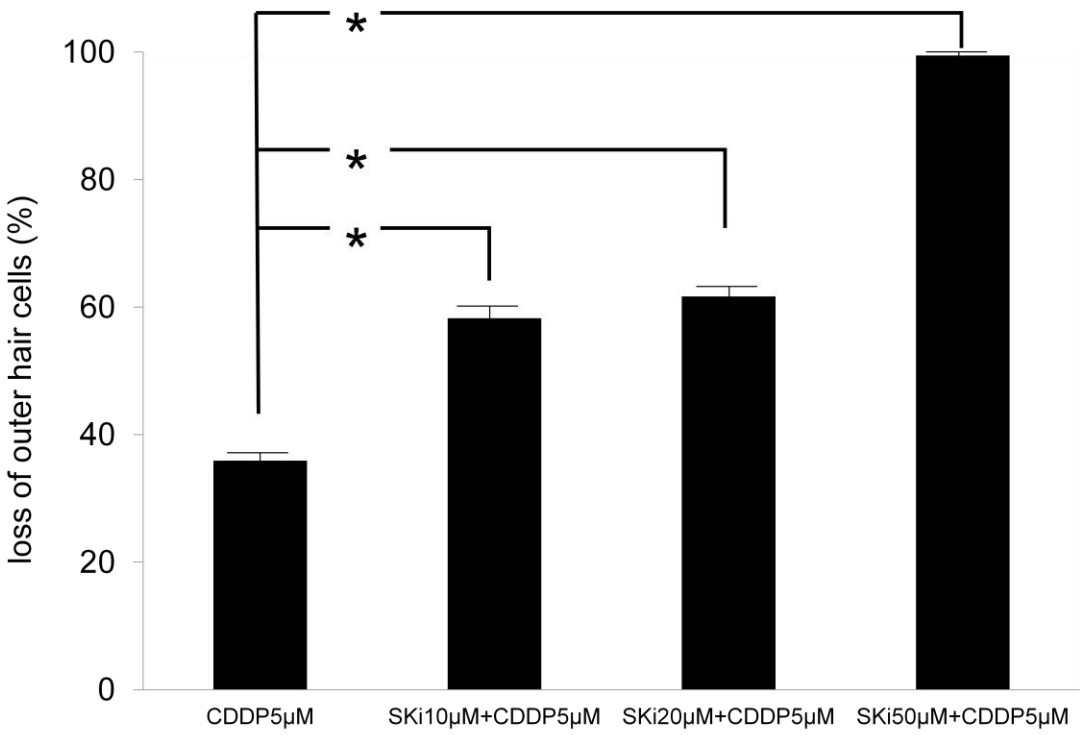
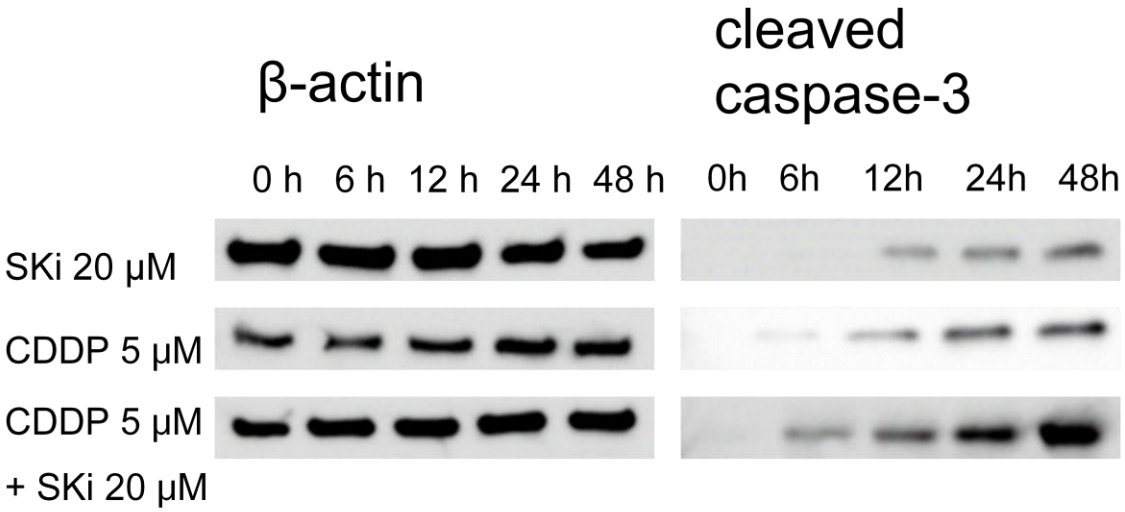

loss of outer hair cells (%)
100
80
60
40
20
0
*
*
*
CDDP5μM
SKi10μM+CDDP5μM
SKi20μM+CDDP5μM
SKi50μM+CDDP5μM

Fig. 6

(A)


β-actin
cleaved caspase-3
0 h 6 h 12 h 24 h 48 h
0h 6h 12h 24h 48h
SKi 20 μM
CDDP 5 μM
CDDP 5 μM
+ SKi 20 μM

(B)

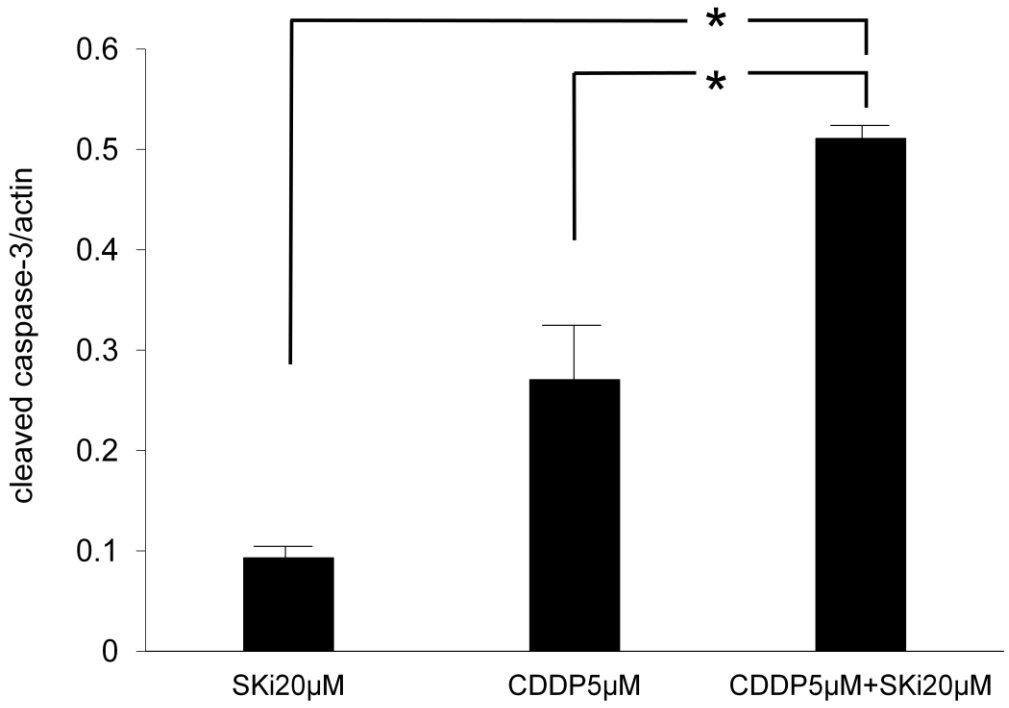
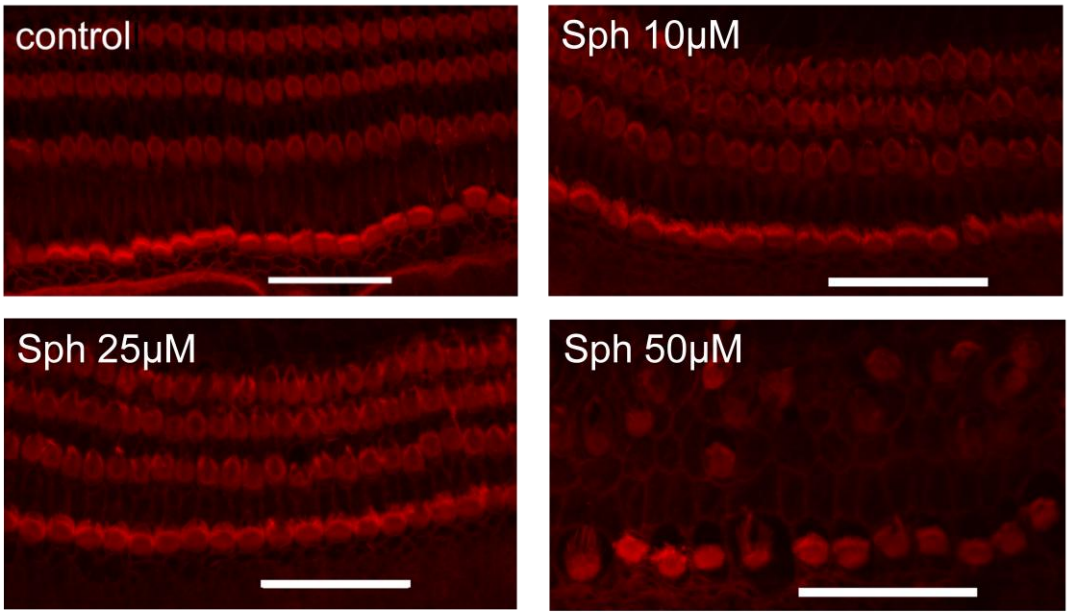

cleaved caspase-3/actin
0.6
0.5
0.4
0.3
0.2
0.1
0
*
*
SKi20μM
CDDP5μM
CDDP5μM+SKi20μM

Fig. 7

(A)


control
Sph 10μM
Sph 25μM
Sph 50μM

(B)

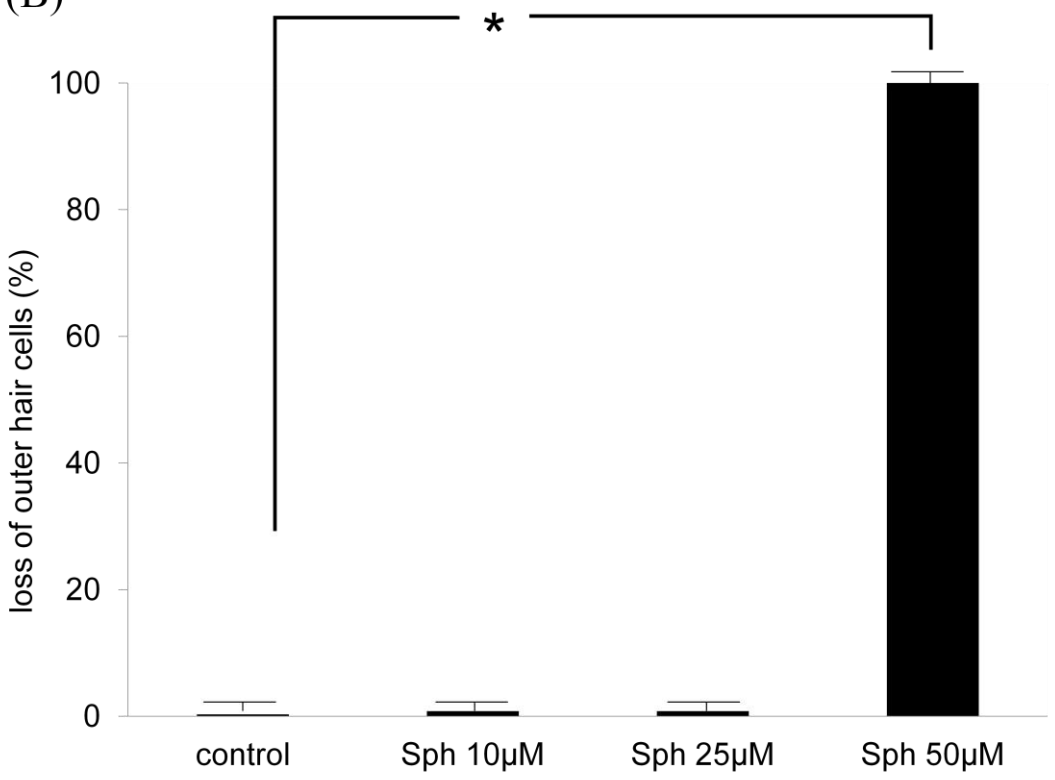
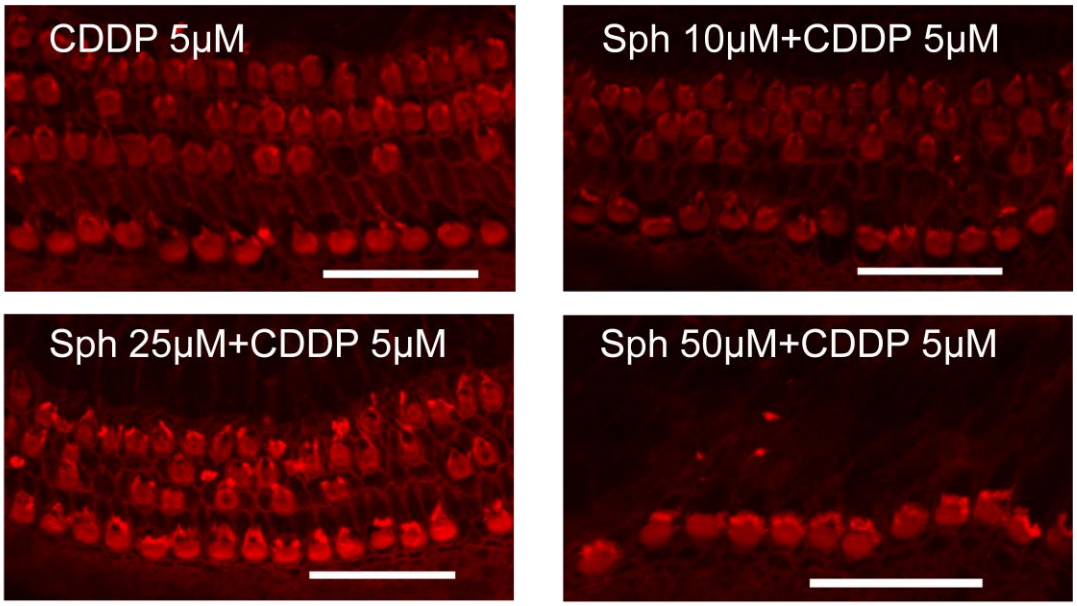

*
100
80
60
40
20
0
loss of outer hair cells (%)
control
Sph 10μM
Sph 25μM
Sph 50μM

Fig. 8

(A)


CDDP 5μM
Sph 10μM+CDDP 5μM
Sph 25μM+CDDP 5μM
Sph 50μM+CDDP 5μM

(B)

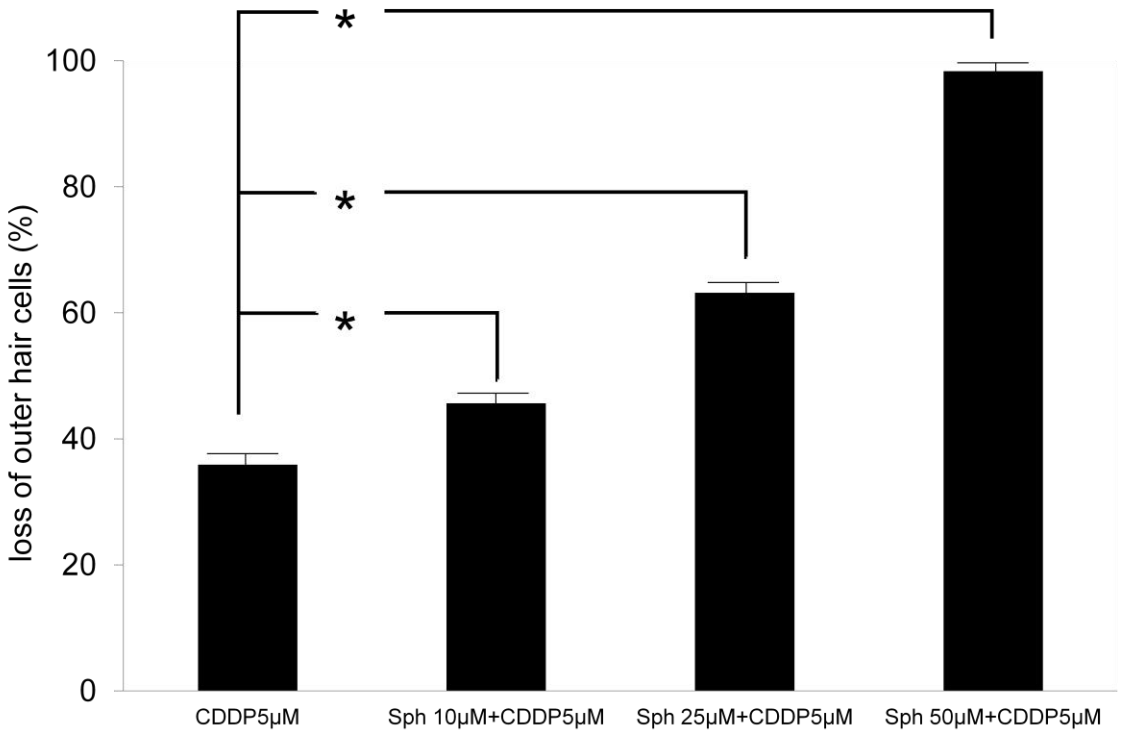
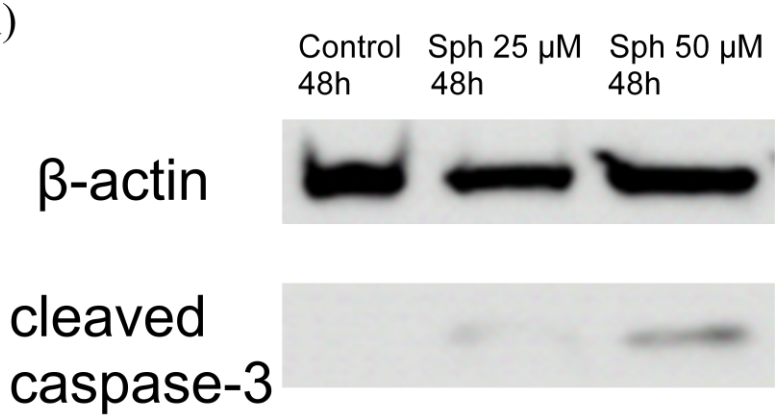

loss of outer hair cells (%)
100
80
60
40
20
0
*
*
*
CDDP5μM
Sph 10μM+CDDP5μM
Sph 25μM+CDDP5μM
Sph 50μM+CDDP5μM

Fig. 9

(A)


Control
48h
Sph 25 μM
48h
Sph 50 μM
48h
β-actin
cleaved
caspase-3

(B)

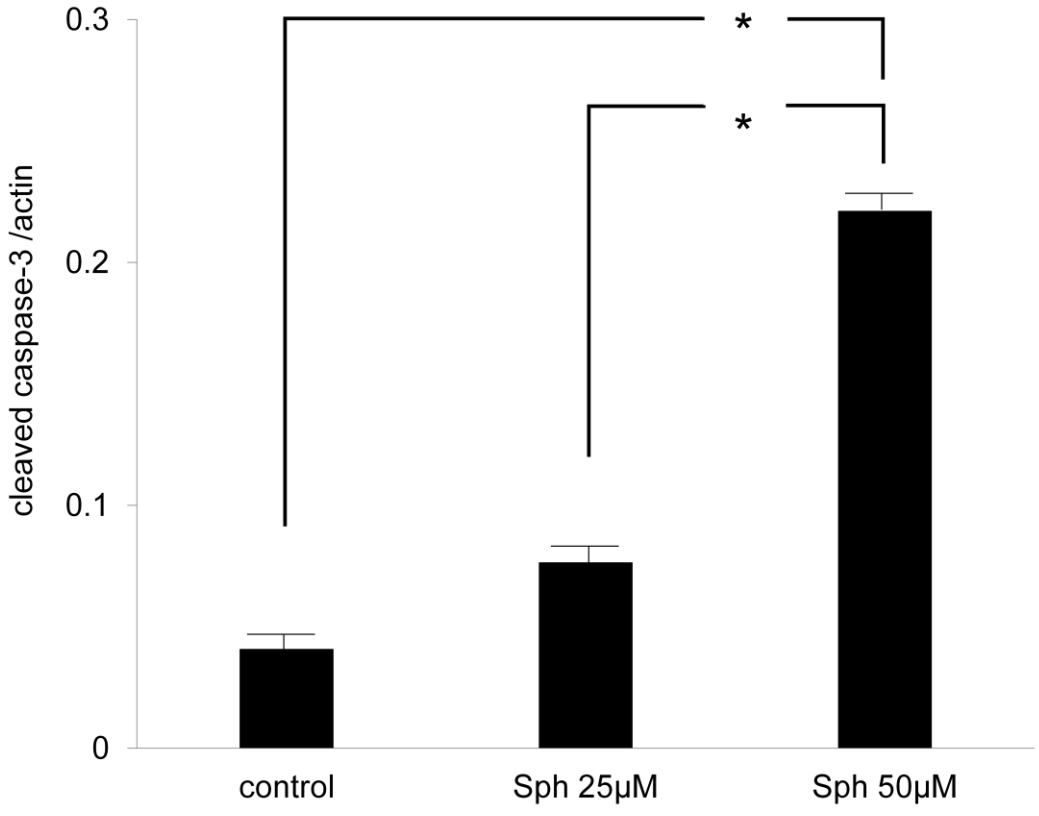
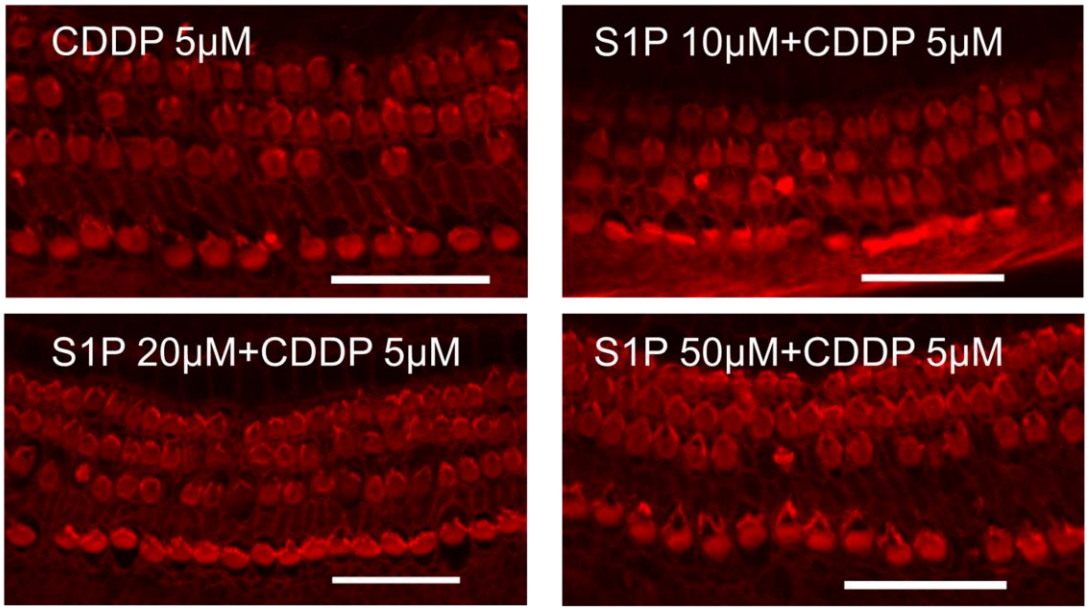

0.3
0.2
0.1
0
cleaved caspase-3 /actin
*
*
control
Sph 25μM
Sph 50μM

Fig. 10

(A)


CDDP 5μM
S1P 10μM+CDDP 5μM
S1P 20μM+CDDP 5μM
S1P 50μM+CDDP 5μM

(B)

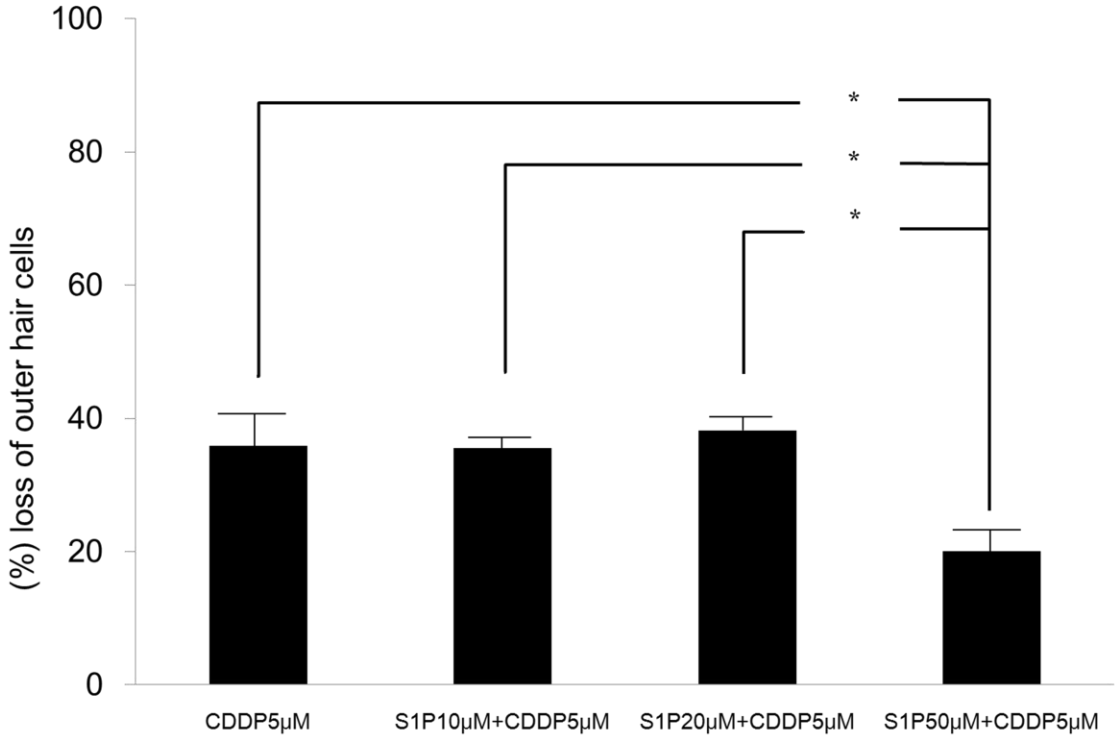

100
80
60
40
20
0
(%) loss of outer hair cells
*
*
*
CDDP5μM
S1P10μM+CDDP5μM
S1P20μM+CDDP5μM
S1P50μM+CDDP5μM

Fig. 11

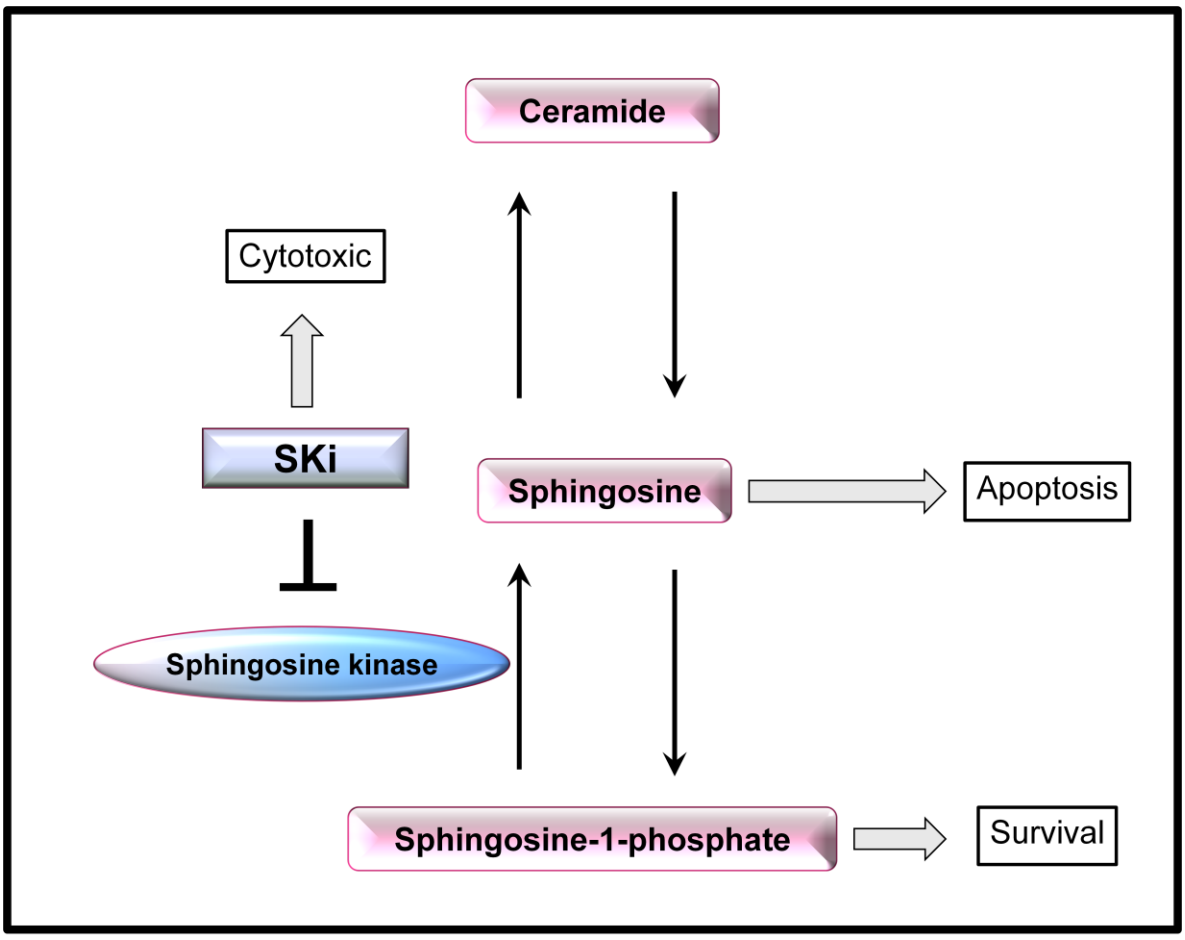

Ceramide
Cytotoxic
SKi
Sphingosine
Apoptosis
Sphingosine kinase
Sphingosine-1-phosphate
Survival

Fig. 12