筑　波　大　学

博 士 （医 学） 学 位 論 文

Otoprotection in Cisplatin-Induced Ototoxicity via Ceramide – 1 – Phosphate and Peroxiredoxin I

（セラミド-1-リン酸、ペルオキシレドキシン 1 によるシスプラチン耳毒性における内耳保護）

2016

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Table of Contents

Abbreviations

Actb	beta-actin
Akt	a serine/threonine-specific protein kinase
ANOVA	analysis of variance
Bcl-2	B-cell lymphoma 2
C1P	ceramide – 1 – phosphate
CDDP	cisplatin
cDNA	complementary deoxyribonucleic acid
CERK	ceramide kinase
Ct	threshold cycle
DEPC	diethylpyrocarbonate
DMEM	Dul-becco’s modified Eagle’s medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ERK	extracellular signal–regulated kinases
FBS	fetal bovine serum
GAPDH	glyceraldehyde-3-phoshate dehydrogenase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IAP	Inhibitors of apoptosis proteins
IHC	inner hair cell
kDa	kilodalton
mRNA	messenger RNA

NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
OHC	outer hair cell
P3 – P5	postnatal day 3 – postnatal day 5
PBS	phosphate-buffered saline
PC	pillar cells
PI3-K	phosphatidyl-inositol-3-kinase
PKC	protein kinase C
PP2A	protein phosphatase 2
Prx	peroxiredoxin
ROS	reactive oxygen species
RNA	ribonucleic acids
RT-PCR	reverse transcription polymerase chain reaction
UV	ultraviolet
WT	wildtype

Chapter 1. General overview

1.1. Structures related to hearing function

Understanding of the anatomy and physiology of cochlea is a better way to learn about mechanisms involved in ototoxicity and to develop a better prevention. For the purpose of our studies, we describe some vital structures of cochlea relating to hearing functions in this chapter. They include cochlear hair cells and the lateral wall (Fig. 1.1). Cochlear hair cells lie along the turns of the cochlea on basilar membrane. There are two types of cochlear hair cells: inner hair cells (IHCs) and outer hair cells (OHCs) housed in the organ of Corti. The inner hair cells are responsible for transferring the fluid motion into neural signal that can be passed to the brain. The OHCs are responsible for modifying the incoming signal. The lateral wall houses structures which are important in the production of the endocochlear potential. The endocochlear potential is a standing potential that exists between the endolymphatic space and the perilymphatic space. Spiral ligament fibrocytes of lateral wall is responsible for active accumulation of potassium ions (K+) in the endolymphatic space (v. Békésy 1952). The endocochlear potential and high endolymphatic K+ are important for K+ entry and triggering of an action potential in cochlear hair cells when ion channels in these cells are opened. The influx of K+ ions ends in changes in cell shape and then release of synaptic vesicles into the synaptic cleft at the base of cochlear hair cell. Although mentioned structures are essential in hearing function, they are very sensitive to harmful stimuli (such as loud sounds, aminoglycosides, or chemotherapy …), and their damages result in hearing loss. In our research, we focus on cisplatin-induced ototoxicity for the purpose of better understating in otoprotection.

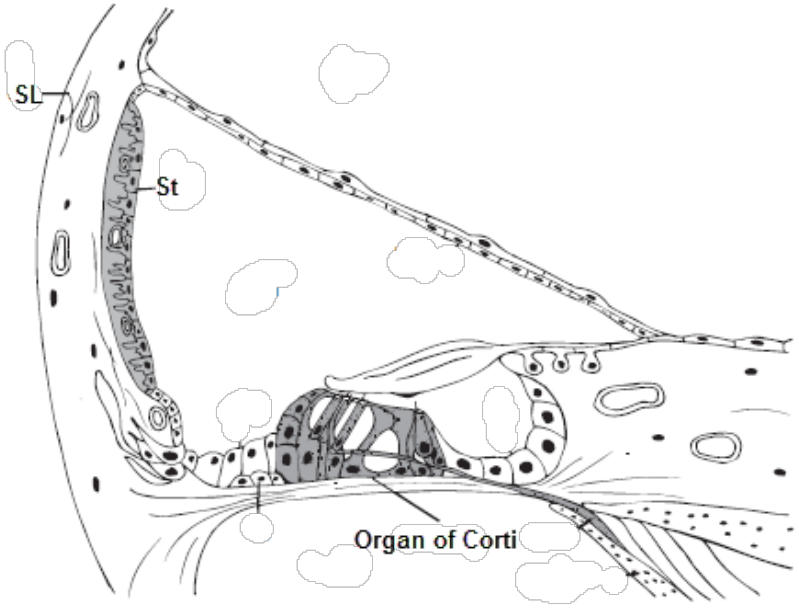


Figure 1.1: Organ of Corti housing of OHC and IHC and lateral wall housing of spiral ligament (SL) and stria vascularis (St)

1.2. Cisplatin and ototoxicity

Cisplatin is an effective anti-neoplastic drug for the treatment of solid tumors (Boulikas and Vougiouka 2004), and is the central component of several curative approaches for patients with head and neck cancer (Lamont and Vokes 2001). However, of all the drugs used in the modern chemotherapy of cancer, cisplatin occupies a unique role in causing significant ototoxicity in a large percentage of patients treated. Importantly, once the incident happens, most investigators have reported virtually no recovery in the ototoxic hearing loss following cessation of cisplatin treatment. Thus, cisplatin ototoxicity is regarded as permanent, and is linked to the degeneration of cochlear OHCs, components of the later wall, as well as spiral ganglion cells (Rybak, Whitworth et al. 2007). Recent experiment research showed that strategies for prevention of cisplatin ototoxicity could

be achieved by either reduction of free radical formation or manipulation of signal transduction pathway to increase survival and decrease apoptosis pathways (Tabuchi, Nishimura et al. 2011). Based on those fundamental knowledge, our research were conducted and results were presented in next chapters to shed more light on cisplatin ototoxicity and its protectors.

Chapter 2. Ceramide-1-phosphate protection of cochlear hair cells against cisplatin ototoxicity

2.1. Introduction

Ceramide, composing of sphingosine and a fatty acid, plays a central role in sphingolipid biosynthesis and catabolism. Ceramide is generally formed either via de novo synthesis from serine and palmitoyl-CoA involving the action of the enzymes serine palmitoyltransferase and ceramide synthetase, or via hydrolysis of sphingomyeline at the plasma membrane by sphingomyelinase (Brown and London 1998, Kolesnick, Goni et al. 2000) (Fig. 2.1). As a bioactive lipid, ceramide has been implicated in a variety of physiological or pathophysilogical functions including apoptosis and cell growth arrest, differentiation, senescence, migration, and adhesion (Hannun and Obeid 2008). To achieve this, ceramides modulate diverse signal transduction pathways by activating or inhibiting key regulatory enzymes including protein phosphatases (Dobrowsky and Hannun 1992), phospholipase D (Gomez-Munoz, Martin et al. 1994), protein kinase C (Berra, Diaz-Meco et al. 1995), specific serine/threonine kinases (Kolesnick and Fuks 1995), stress-activated protein kinases also known as Jun nuclear kinases (Verheij, Bose et al. 1996), mitogen-activated protein kinases (Spiegel, Foster et al. 1996), phospholipase A2 (Hayakawa, Jayadev et al. 1996), and protein kinase B (Bourbon, Sandirasegarane et al. 2002). Roles for ceramide as a cell-death-inducing factor have also been suggested in a number of pathological conditions including cancer, neurodegeneration, diabetes, microbial pathogenesis, obesity, and inflammation (Wu, Ren et al. 2007, Zeidan and Hannun 2007).

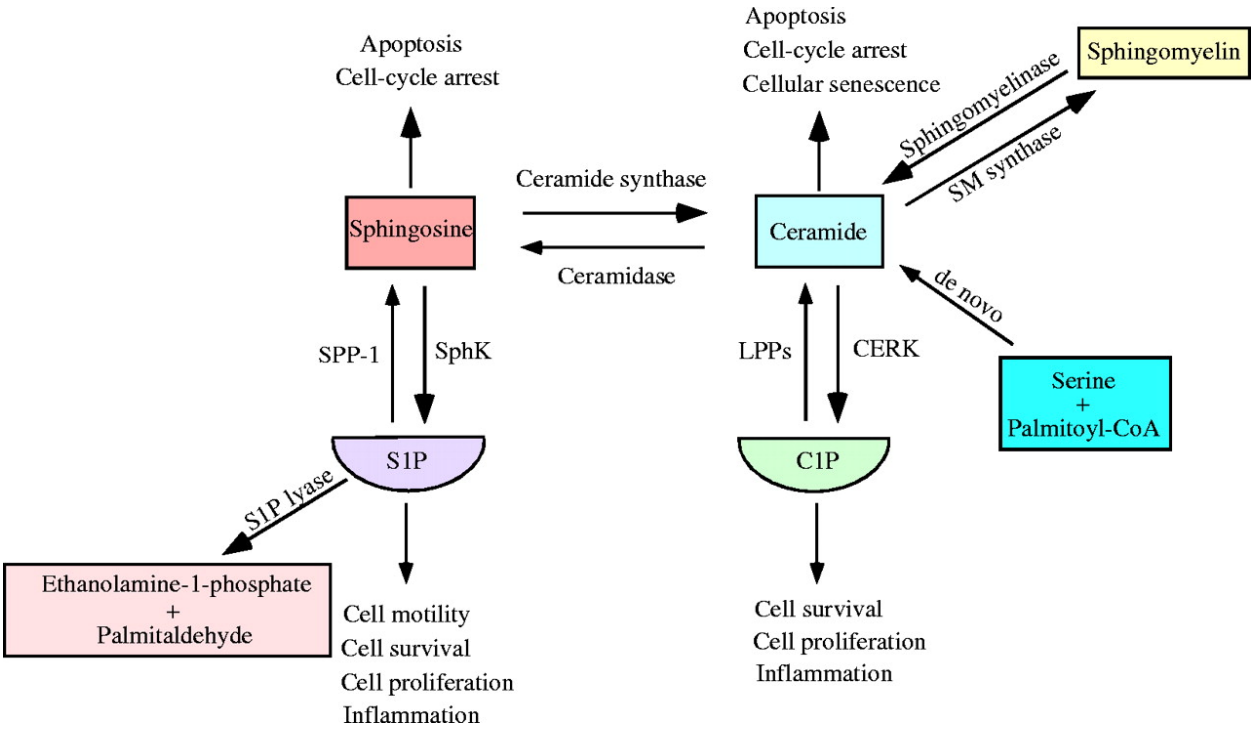


Figure 2.1: bioactive sphingolipid metabolites (Chalfant and Spiegel 2005)

(SPP-1: S1P phosphatase-1; SphK: sphingosine kinase; S1P: sphingosine 1-phosphate; LPP: lipid phosphate phosphatase; CERK: ceramide kinase; C1P: ceramide – 1- phosphate; SM: sphingomyelin)

In the sphingomyelin cycle, a number of extracellular agents and insults (such as tumor necrosis factor, Fas ligands, and chemotherapeutic agents) cause the activation of sphingomyelinases, which acts on membrane sphingomyelin and releases ceramide. In the relation to cisplatin-induced apoptosis of cochlear hair cells, increase of ceramide was identified as one of decisive factors. It was demonstrated that cisplatin activates sphingomyelinase, which triggers the release of ceramide (Lacour, Hammann et al. 2004). Consequently, ceramide sensitizes cells to apoptosis by transforming cellular-membrane properties (Veiga, Arrondo et al. 1999, Megha and London 2004), blocking phosphatidyl-

inositol-3-kinase (PI3-K) (Zundel, Swiersz et al. 2000), and inducing death receptors to transmit strong signals (Carpinteiro, Dumitru et al. 2008). Although ceramide is known to be an inducer of apoptosis in aminoglycoside ototoxicity (Nishimura, Tabuchi et al. 2010), the effects of ceramides on cisplatin ototoxicity have never been examined before.

Ceramide-1-phosphate (C1P) is synthesized by phosphorylation of ceramide in mammalian cells (Dressler and Kolesnick 1990), and currently, ceramide kinase (CERK) is the only known mammalian enzyme to have this function. It was firstly found in human leukemia cells (HL-60). Later, researchers observed that endogenous C1P can be generated during the phagocytosis of antibody-coated erythrocytes in human neutrophils (Hinkovska-Galcheva, Boxer et al. 1998). Also, C1P can also be generated by the action of interleukin 1-β on A549 lung carcinoma cells (Pettus, Bielawska et al. 2003). More recently, C1P was found presented in normal bone marrow-derived macrophages isolated from healthy mice (Gomez-Munoz, Kong et al. 2004). Since the first biological activity of C1P relating to DNA synthesis and cell division was identified (Gomez-Munoz, Duffy et al. 1995, Gomez-Munoz, Frago et al. 1997), researchers have found that this phospholipid was attributed to various vital functions, such as macrophage proliferation and migration (Granado, Gangoiti et al. 2009, Gangoiti, Granado et al. 2010), phagocytosis (Hinkovska-Galcheva, Boxer et al. 1998), inflammation (Pettus, Bielawska et al. 2003, Pettus, Bielawska et al. 2004) and cell protection (Gomez-Munoz, Kong et al. 2004, Gomez-Munoz, Kong et al. 2005). However, it has not been revealed yet whether C1P could inhibit cochlear hair cell death induced by cisplatin.

Induction of cell death is a complex process and tightly regulated. In the present study we hypothesized that C1P could be an antiapoptotic molecule and that targeted ceramide – C1P balance could interfere cochlear cell survival.

2.2. Methods

2.2.1. Culture technique

2.2.1.1. Cochlear explants

The lower basal turn of the organ of Corti was dissected from C57BL/6J mouse on postnatal days 3 (P3) to 5 (P5) and cultured according to the methods of Van de Water and Ruben (Van de Water and Ruben 1974) and Sobkowicz et al. (Sobkowicz, Loftus et al. 1993). In brief, after sterilized, a mice pup were decapitated. The ear capsule was quickly exposed and taken out. The organ of Corti housing cochlear hair cells was isolated from other structure and was transferred to culture media. All animal procedures were carried out according to the guidelines of the Laboratory Animal Research Center of Tsukuba University (Fig. 2.2)

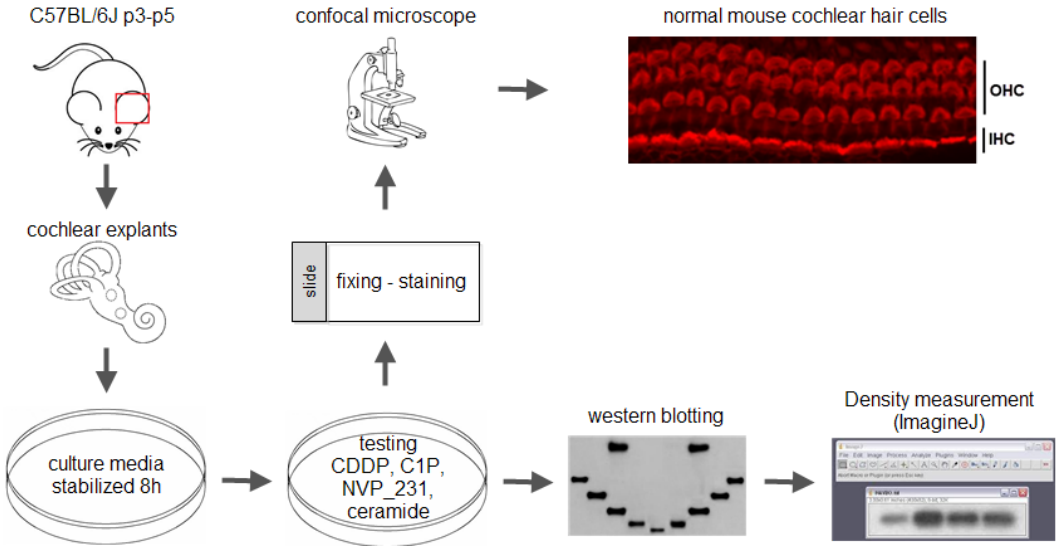


Figure 2.2. Scheme of experimental design

2.2.1.2. Culture techniques

The organs of Corti (cochlear explants) were maintained in a culture medium containing Dul-becco's modified Eagle's medium (DMEM), 10% fetal bovine serum (FBS), 25 mM HEPES, and 30 U/mL penicillin. They were cultured in an incubator at 37^{o}C with 5% CO2 at 95% humidity. Cochlear explants were maintained in culture medium overnight (8 – 12 hours) and then were exposed to a new culture medium containing tested agents (e.g. cisplatin, C1P, or NVP-231 …) for 48 hours (Tabuchi, Pak et al. 2007).

2.2.1.3. Cisplatin treatment

Cisplatin (Nichi-Iko, Sogawa, Japan) was used in a form of commercial liquid. Cisplatin-induced ototoxicity was characterized by the missing of cochlear OHCs at the basal turn of the organ of Corti. Before conducting this study, we examined the damage of cochlear OHCs by exposing cochlear explants to several concentrations of cisplatin from 1 to 50 μM. The concentrations of cisplatin at 5 and 10 μM were chosen for the present experiments.

2.2.1.4. C1P treatment

C1P (Sigma, St. Louis, MO, USA) was initially dissolved in ethanol at a concentration of 2 mg/ml, and was routinely stored at -20 ^{o}C. Before used, C1P was diluted in the culture medium to the final concentrations. After the cochlear explants were stabilized in the culture medium overnight, each group (from 9 to 18 cochlear explants) was exposed to culture media containing 10 μM cisplatin plus various concentrations of C1P (from 1 to 100 μM) for 48 hours.

2.2.1.5. Ceramide treatment

C16-ceramide (LKT Laboratories, St. Paul, MN, USA) was dissolved in ethanol at a concentration of 5 mg/ml and was stored at -20 ^{o}C. The effect of ceramide alone on cochlear explants was tested using concentrations from 10 to 500 μM. Later, the combination of 5 μM cisplatin and various concentrations of ceramide (from 10 to 500 μM) was examined. In another condition, 500 μM ceramide was selected to mix with various concentrations of NVP-231 (from 10 to 200 μM), and this combination was applied to assess the survival of cochlear OHCs. In all experiments, cochlear explants were stabilized in the normal culture medium overnight before treated in different conditions for 48 hours.

2.2.1.6. NVP-231 treatment

NVP-231 was dissolved in dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO, USA) at a concentration of 10 mg/ml, and was stored at 4 ^{o}C. Control experiments were conducted by treating cochlear hair cells with NVP-231 ranging from 10 to 200 μM. Next, cochlear explants, which were stabilized in the culture medium overnight, were exposed to culture media containing 5 μM cisplatin plus various concentrations of NVP-231 (from 0.1 to 10 μM) for 48 hours.

2.2.2. Cytochemistry

At the end of the tissue culture, cochlear 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,USA) in PBS with 10% fetal

bovine serum for 10 minutes. The specimens were stained with phalloidin using a conjugated Alexa Fluor probe (1:100, Molecular Probes, Carlsbad, CA, USA) at room temperature for 1 hour (Tabuchi, Pak et al. 2007). Phalloidin is a specific marker for cellular F-actin and labels stereociliary arrays and the cuticular plates of hair cells. The specimens were observed using a confocal microscope (FluoView F10i, Olympus, Center Valley, PA, USA). Hair cells were characterized as missing if no stereocilia or no cuticular plates were observed by phalloidin staining. Quantitative results were obtained by evaluating 30 OHCs associated with 10 inner hair cells in a given microscopic field. The average of three randomly separated counts was used to represent each tissue culture. Residue of cochlear OHCs was expressed as a percentage and the results of each the groups were compared (Tabuchi, Pak et al. 2007).

2.2.3. Western blots

After collected using the same methods described above, the organs of Corti were treated in culture media alone or in culture media containing either 10 μM cisplatin or 10 μM cisplatin plus 100 μM C1P for 24 or 48 hours. Explants were then homogenized in Pro-Prep protein extraction solution (iNtRON, Kyungki, Korea) following the product's protocol. After centrifuged at 13,000 rpm at 4^{o}C for 10 minutes, the supernatant was transferred to a fresh tube and the protein content was measured by using protein assay rapid kit (Wako, Osaka, Japan). Equal amounts of lysate protein (20 μg) 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 ^{o}C for 5 min, and then subjected to SDS–PAGE using 4%-20% mini-PROTEAN TGX precast polyacrylamide gel

(Bio-Rad, Hercules, CA, USA). The proteins were transferred by means of a semidry electroblotting system from the gels to Hybond polyvinylidene difluoride membrane (GE Healthcare, Buckinghamshire, UK) for 1 hour. Skim milk powder was used for blocking buffer. After blocking, each membrane was primarily treated with polyclonal antibodies Akt (1:500), pAkt (1:500), ERK1/2 (1:500), or pERK1/2 (1:500) overnight at 4ºC. Horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (1:2000) was secondarily treated for 1 hour at room temperature. The antibodies described above were purchased from Cell Signaling Technology (Beverly, MA, USA). Chemiluminescent detection was enhanced with ECL kit (GE Healthcare, Buckinghamshire, UK) and the immunoblot images were obtained with the ImageQuant LAS4000 mini imager (GE Healthcare, Buckinghamshire, UK). Densitometric analysis was done from at least 3 independent experiments by using ImageJ software (Nation Institutes of Health).

2.2.4. Data analysis

All data were expressed as the means ± SDs. Statistical analysis was performed by t-test or one-way analysis of variance (ANOVA) followed by Bonferroni post hoc tests, as required (SPSS 20). Probability values less than 0.05 were considered significant.

2.3. Results

2.3.1. Control study

A series of concentrations from 2 to 50 μM was selected to test the ototoxicity of cisplatin on OHCs. The results showed that there were significant differences among cisplatin groups (Fig. 2.3; ANOVA followed by Bonferroni post hoc test; $P < 0.05$ among

groups), and it also suggested that cisplatin has a dose-dependent effect on OHCs. From the obtained data, concentrations of 5 and 10 μM were selected for next appropriated purposes of this study.

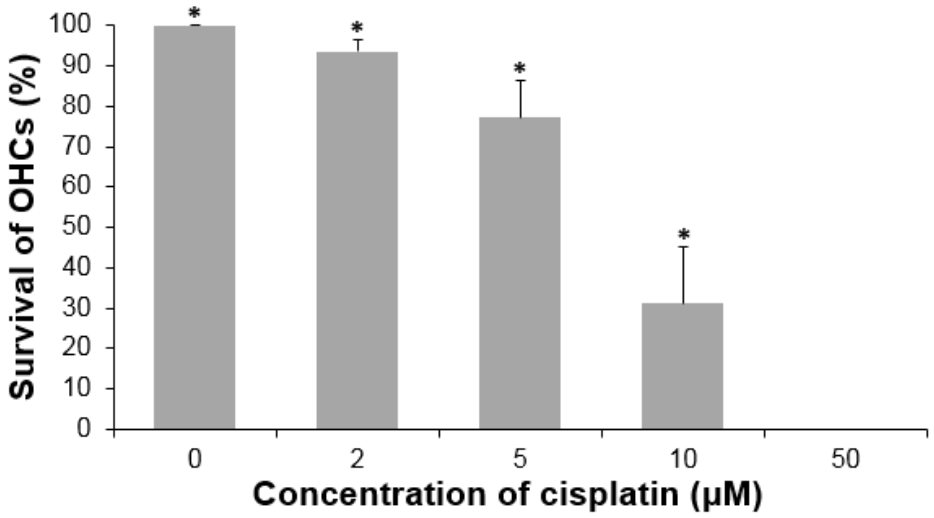
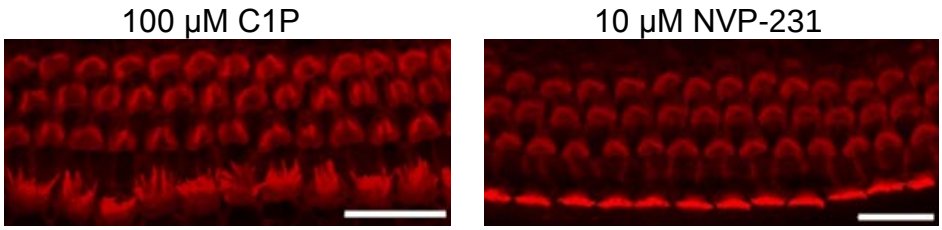


Figure 2.3. Effect of different concentrations of cisplatin on cochlear OHCs. The survivals of cochlear OHCs were around 93.5 %, 77.1%, and 31% corresponding to 2, 5, and 10 μM cisplatin, respectively. Cochlear hair cells were completely destroyed at 50 μM cisplatin. (ANOVA followed by Bonferroni post hoc test, P <0.05)

The effects of 100 μM C1P, 10 μM NVP-231 or solvents alone (1% ethanol and 0.04% DMSO) on cochlear OHCs were examined. After 48-hour exposure to above agents, all of cochlear OHCs remained intact. (Fig. 2.4)



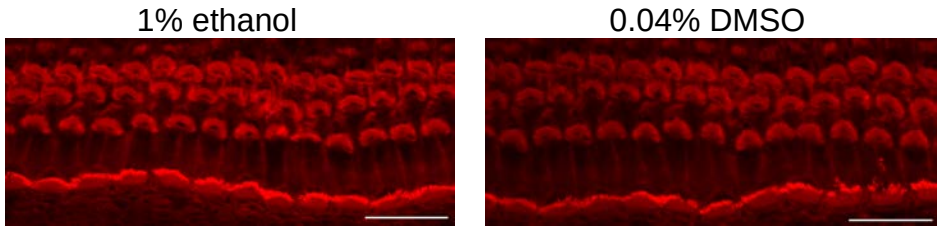


Figure 2.4. Effect of C1P, NVP-231 and solvent on cochlear OHCs

In addition, the interactions between cisplatin and solvents were also checked. There were no significant differences in cochlear hair cell loss between explants treated with cisplatin alone and one treated with cisplatin plus either 1% ethanol or 0.04% DMSO; the tested concentrations of cisplatin were 10 μM (Fig. 2.5)

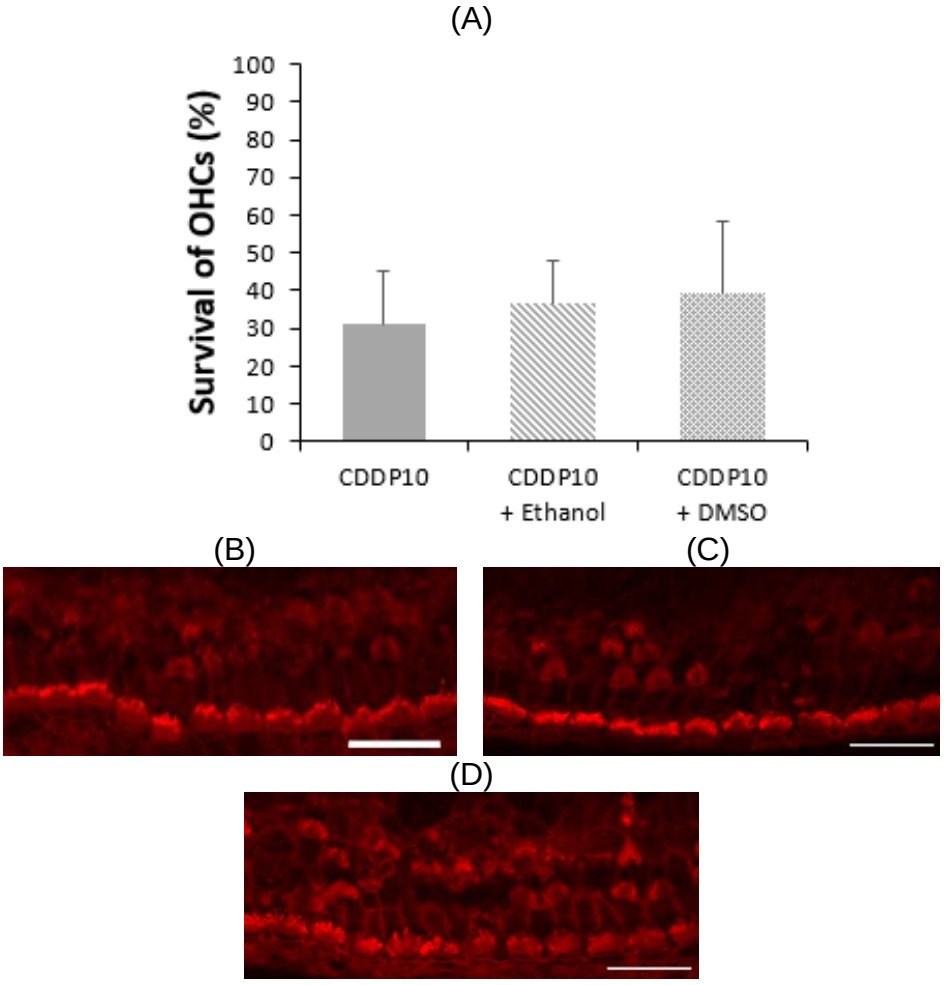
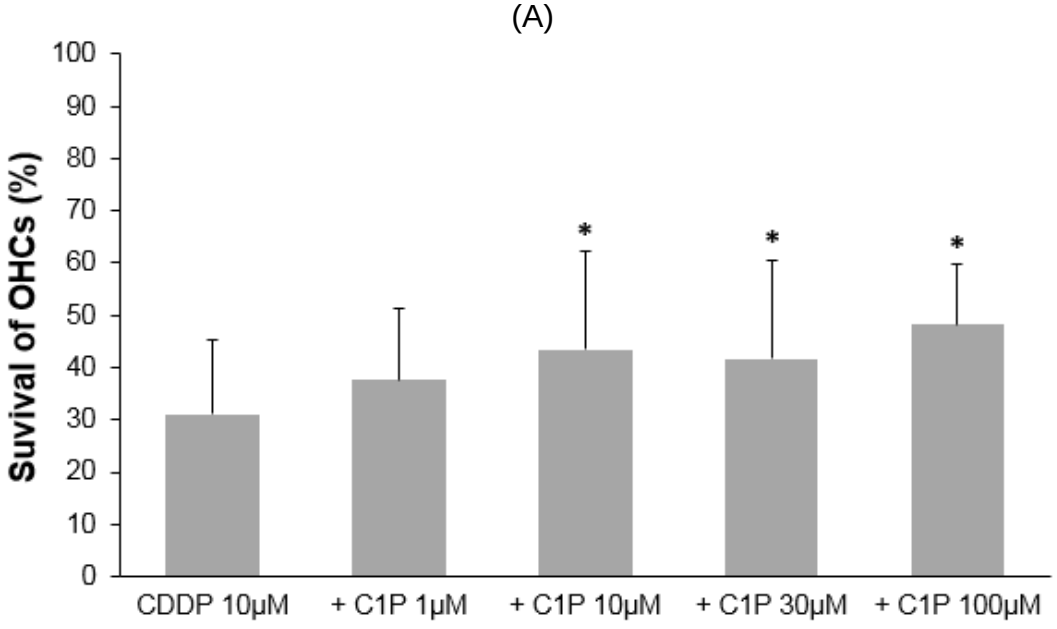


Figure 2.5. Effect of cisplatin with the addition of solvents.

(A) Quantitative analysis of cochlear OHCs (ANOVA followed by Bonferroni post hoc test, *P* >0.05). (B) 10 μM cisplatin. (C) 10 μM cisplatin plus 1% ethanol. (D) 10 μM cisplatin plus 0.04% DMSO. CDDP: cisplatin

2.3.2. C1P and the survival of OHCs in cisplatin ototoxicity

We examined the effect of C1P on cisplatin-induced OHC damage. The results revealed a dose-dependent relationship between C1P treatment and the survival of cochlear OHCs. When compared with cisplatin treatment alone, the addition of C1P treatment significantly increased the survival of cochlear OHCs at 10 μM C1P or higher (Fig. 2.6; ANOVA followed by Bonferroni post hoc test; 10 μM-C1P group, n = 16, *P* = 0.014; 30 μM-C1P, n = 18, *P* = 0.03; 100 μM-C1P, n = 9, *P* = 0.007; n is the number of cochlear explants).



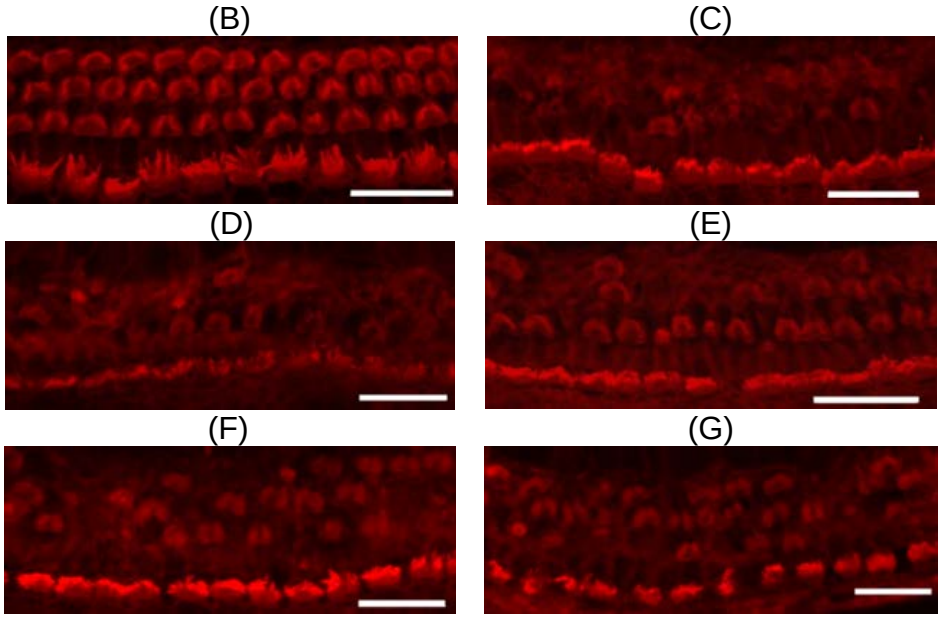


Figure 2.6. Effect of C1P on the survival of cochlear OHCs in cisplatin ototoxicity. (A) Quantitative analysis of cochlear OHCs. The survival of cochlear OHCs significantly increased at 10 μM C1P or higher (ANOVA followed by Bonferroni post hoc test, *P < 0.05). (B – G) Representative photographs of each group (scale bar 20 μm). (B) 100 μM C1P alone. (C) 10 μM cisplatin alone. (D) 10 μM cisplatin and 1 μM C1P. (E) 10 μM cisplatin and 10 μM C1P. (F) 10 μM cisplatin and 30 μM C1P. (G) 10 μM cisplatin and 100 μM C1P. CDDP: cisplatin.

2.3.3. C1P and phosphorylation of Akt and ERK1/2

The above results suggested that C1P could inhibit cochlear OHC death induced by cisplatin. Next, Western blots were conducted to determine whether this effect of C1P involves the stimulation of the PI3-K/Akt or ERK1/2 pathway. Cochlear explants were treated in 3 different conditions, including the culture medium alone, 10 μM cisplatin, or 10 μM cisplatin plus 100 μM C1P. Immunoblot images revealed strong activations of Akt

(Fig. 2.7A, 2.7C) and ERK1/2 (Fig. 2.7E) in cochlear explants treated with C1P. In addition, densitometric analysis also revealed significant increases of pAkt in a group of C1P addition compared to a group without C1P for 24 hours (Fig. 2.7B; t-Test , *P* = 0.005) or 48 hours (Fig. 2.7D; t-Test, *P* = 0.025). The signal of pERK1/2 was also significantly increased when C1P was added for 48 hours (Fig. 2.7F; t-test, *P* = 0.015)

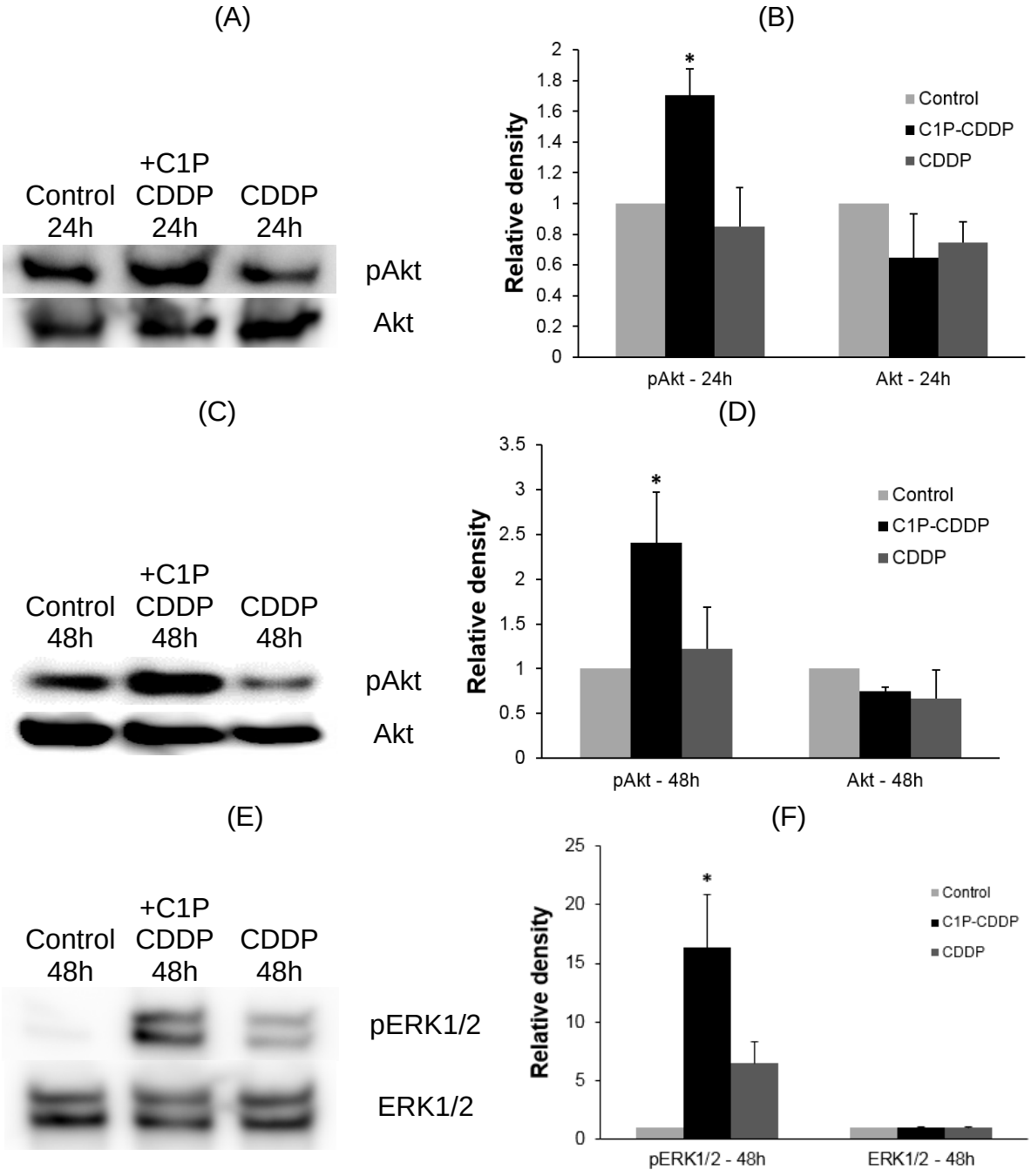


Figure 2.7. Representative Western blots of pAkt, total Akt, pERK1/2 and total ERK1/2. Comparisons were conducted among 3 groups: control group (no cisplatin), 10 μM cisplatin plus 100 μM C1P group, and 10 μM cisplatin group. (A) Chemiluminescent detection of pAkt and Akt with 24-hour treating time. (C) Chemiluminescent detection of pAkt and Akt with 48-hour treating time. (E) Chemiluminescent detection of pERK1/2 and ERK1/2 with 48-hour treating time. (B, D, F) C1P significantly increased densitometric signals of pAkt and pERK1/2 in comparison to groups without C1P (*t*-test, $P < 0.05$). Densitometric values were normalized to values of control group. pAkt and pERK1/2 have been referenced to total Akt and total ERK1/2, respectively. Data were obtained from three independent experiments. CDDP: cisplatin.

2.3.4. The effect of ceramide on cisplatin-induced cochlear OHCs death

Ceramide is a regulator of cellular apoptosis, and this experiment was conducted to show whether or not ceramide amplifies cisplatin-induced hair cell death. First, ceramide alone was examined on cochlear explants. All cochlear OHCs were intact at 200 μM ceramide or lower, and very few hair cells were lost at 500 μM (Fig 2.8). Later, several concentrations of ceramide as above were tested with 5 μM cisplatin addition. Only the condition of 500 μM ceramide plus 5 μM cisplatin significantly decreased the number of cochlear OHCs (Fig. 2.9; ANOVA followed by Bonferroni post hoc test; n = 9, $P < 0.001$ at 500 μM).

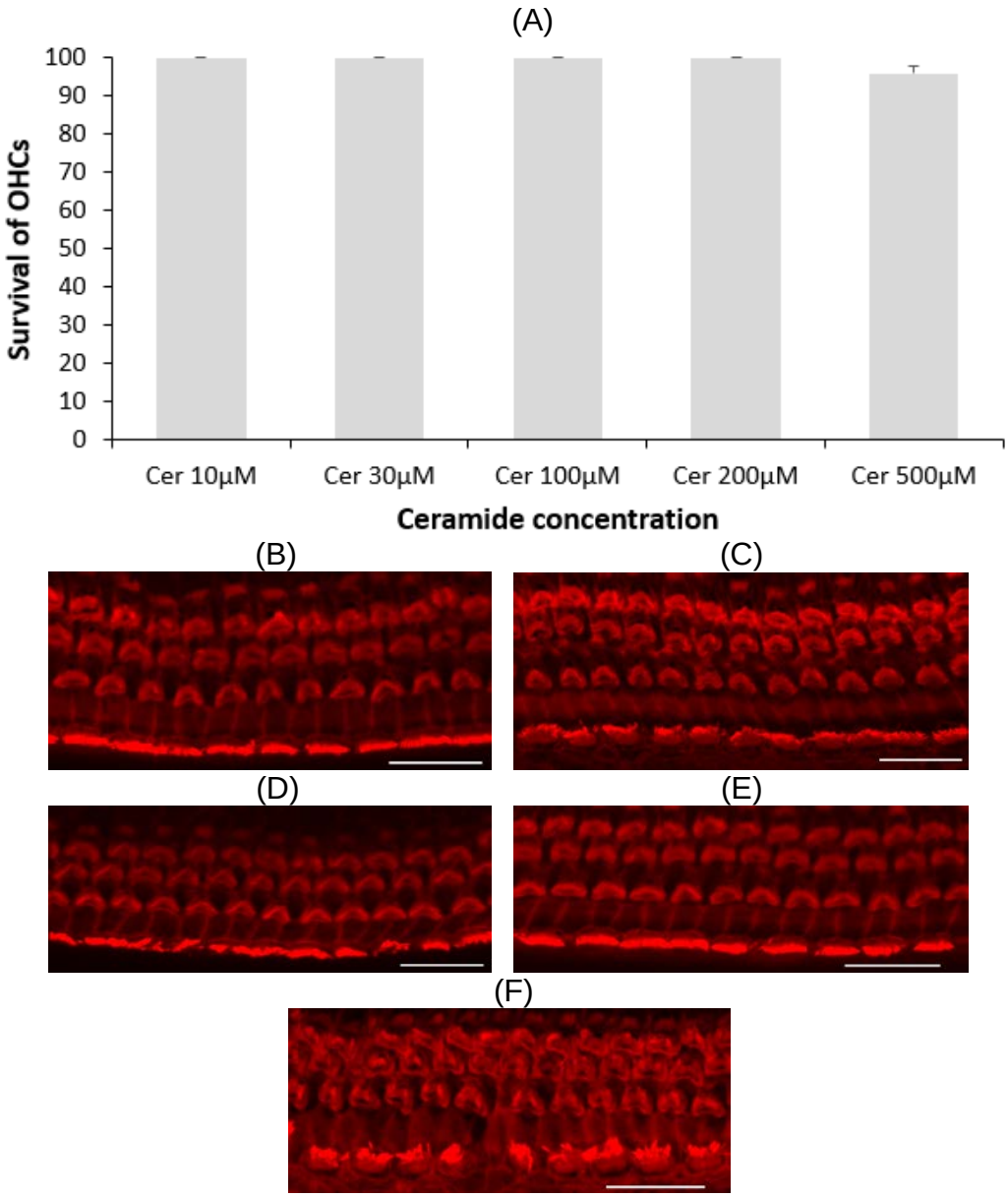


Figure 2.8. Effects of ceramide on cochlear OHCs.

(A) Quantitative analysis of cochlear OHCs. All cochlear OHCs were intact with ceramide concentration lower than 200 μM and few were lost at 500 μM. (B – F) Representative photographs of each group (scale bar 20 μm). (B) 10 μM ceramide. (C) 30 μM ceramide. (D) 100 μM ceramide. (E) 200 μM ceramide. (F) 500 μM ceramide. Cer: ceramide.

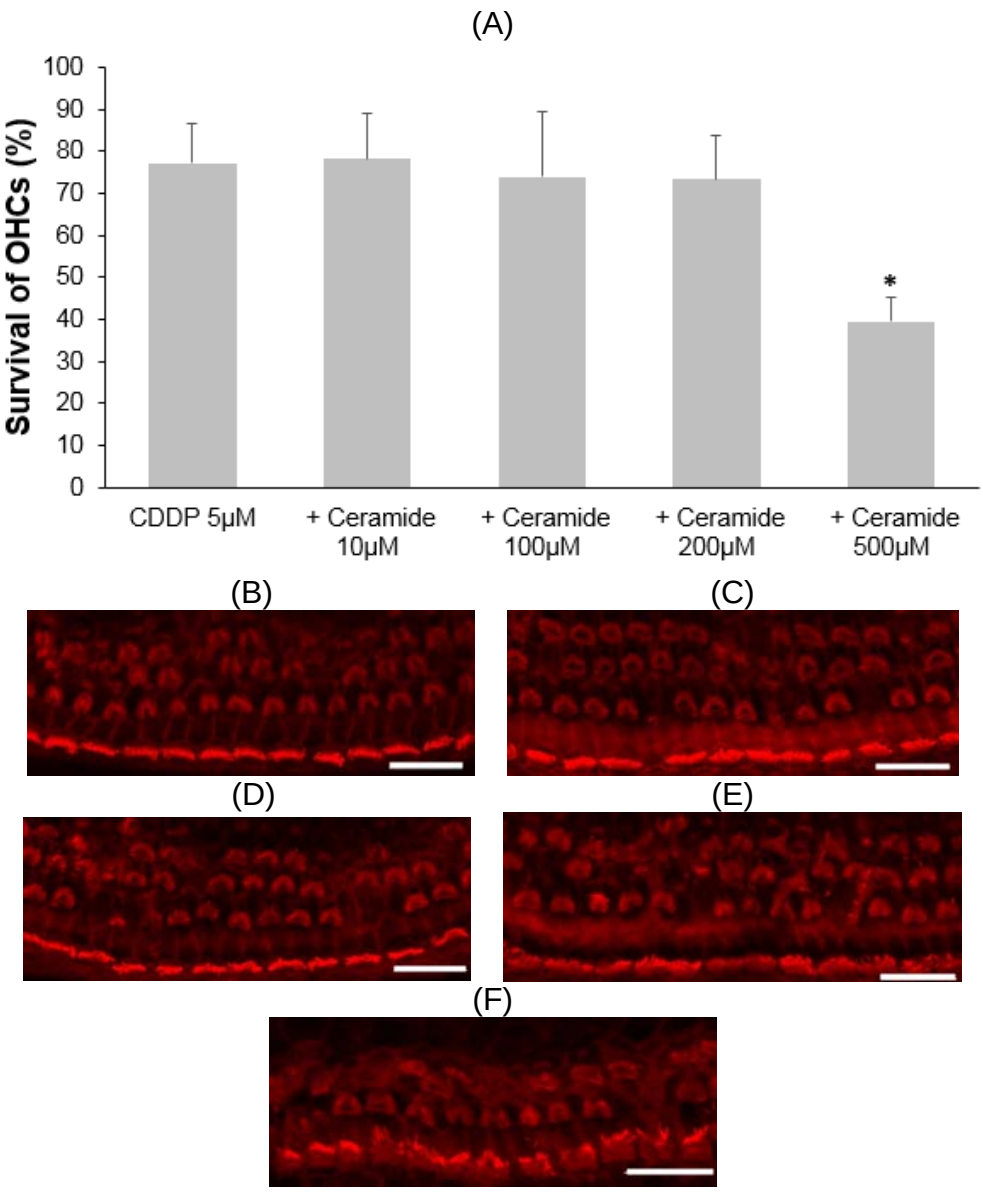


Figure 2.9. Effects of ceramide on cisplatin-induced cochlear OHC death. (A) Quantitative analysis of cochlear OHCs. The residue of cochlear OHCs decreased significantly at 500 μM ceramide (ANOVA followed by Bonferroni post hoc test, $*P < 0.001$). (B – F) Representative photographs of each group (scale bar 20 μm). (B) 5 μM cisplatin alone. (C) 5 μM cisplatin and 10 μM ceramide. (D) 5 μM cisplatin and 100 μM ceramide. (E) 5 μM cisplatin and 200 μM ceramide. (F) 5 μM cisplatin and 500 μM ceramide. CDDP: cisplatin.

2.3.5. CERK inhibitor and cochlear hair cell death induced by cisplatin or ceramide

CERK converts ceramide to C1P and contributes to the balance of the C1P and ceramide levels. NVP-231, a selective CERK inhibitor, was used to evaluate the involvement of CERK when cochlear OHCs exposure to cisplatin or ceramide.

Before conducting experiments in this step, series of concentrations of NVP-231 were tested on cochlear OHCs to evaluate its toxicity. At 10 μM NVP-231, all cochlear hair cells were intact. From 30 to 200 μM NVP-213, very few cochlear hair cells were loss and there were no statistical difference among groups (Fig. 2.10)

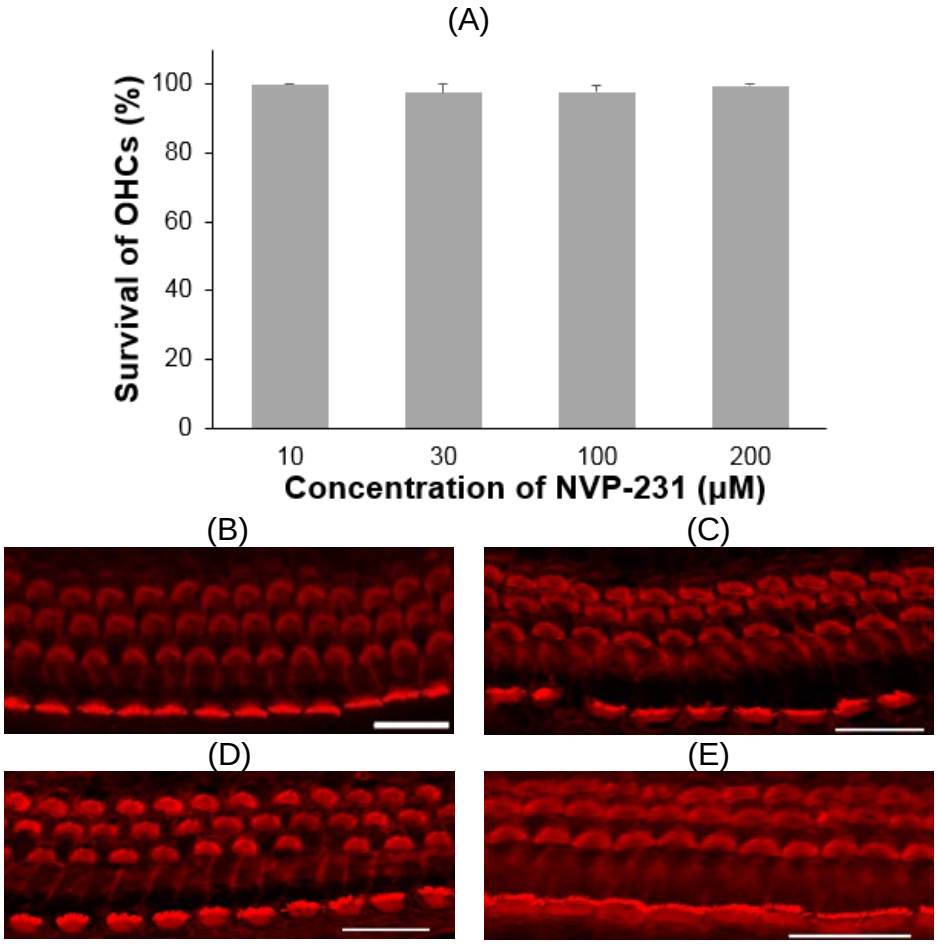
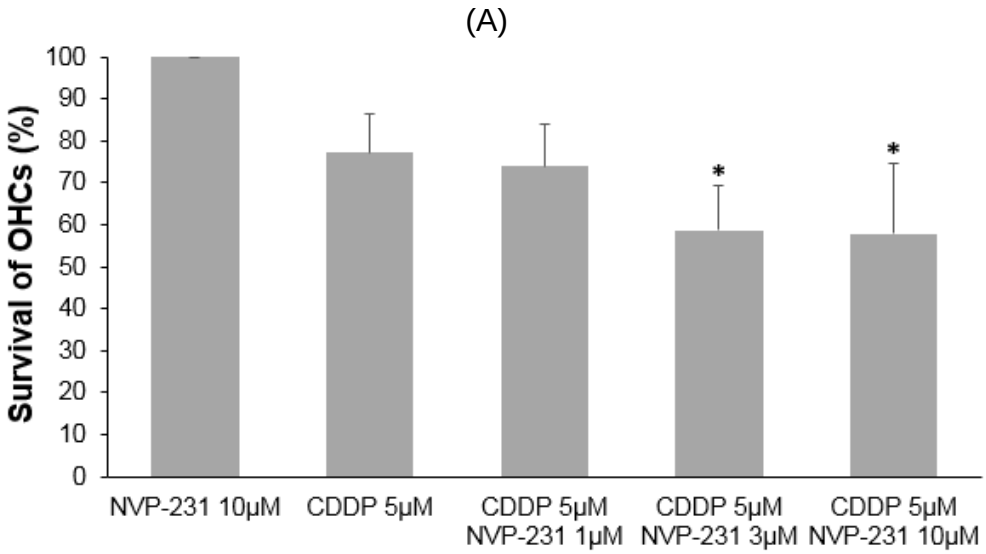


Figure 2.10. Effect of NVP-231 on cochlear OHCs.

(A) Quantitative analysis of cochlear OHCs (ANOVA followed by Bonferroni post hoc test, *P* >0.05). (B) 10 μM NVP-231. (C) 30 μM NVP-23. (D) 100 μM NVP-23. (E) 200 μM NVP-23

At first, 5 μM cisplatin was mixed with to several concentrations of NVP-231. OHC cell death induced by 5 μM cisplatin was significantly exacerbated in the presence of NVP-231 at 3 μM or higher (Fig. 2.11; ANOVA followed by Bonferroni post hoc test; 3 μM NVP-231 group, n = 10, *P* = 0.008; 10 μM NVP-231 group, n = 10, *P* = 0.005). Later, in a different condition, 500 μM ceramide was combined with several concentrations of NVP-231. OHC death was severely damaged, starting with 10 μM NVP-231, and OHCs were completely unable to identify at 200 μM (Fig. 2.12; ANOVA followed by Bonferroni post hoc test; 10 μM NVP-231 group, n = 9, *P* = 0.000; 30 μM NVP-231 group, n = 10, *P* = 0.000; 100 μM NVP-231 group, n = 9, *P* = 0.000)



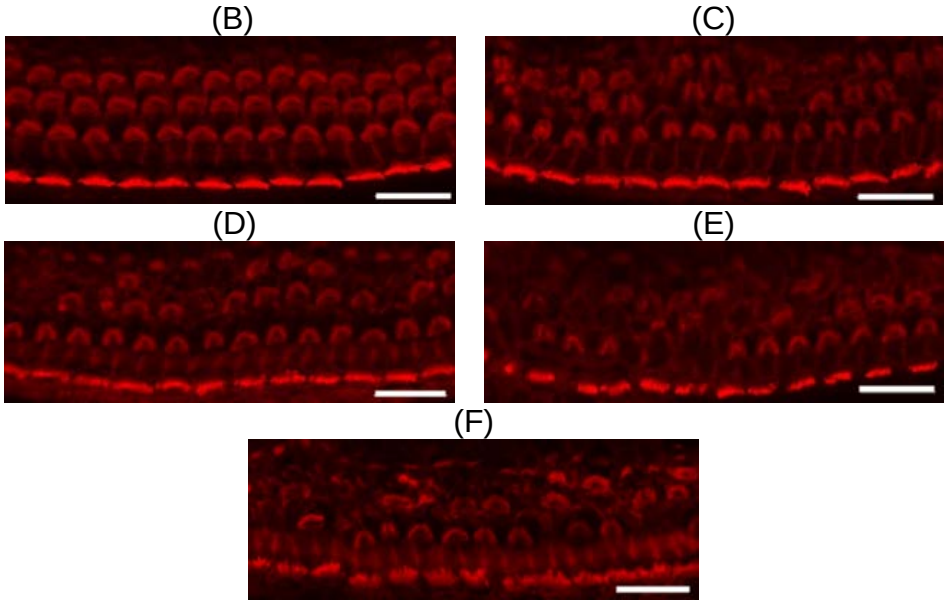


Figure 2.11. Effects of NVP-231 on cisplatin-induced OHC death.

(A) Quantitative analysis of cochlear OHCs. The survival of cochlear OHC was statistically decreased through the effect of 3 μM NVP-231 or higher (ANOVA followed by Bonferroni post hoc test, $*P < 0.05$). (B – F) Representative photographs of each group (scale bar 20 μm). (B) 10 μM NVP-231 alone. (C) 5 μM cisplatin alone. (D) 5 μM cisplatin and 1 μM NVP-231. (E) 5 μM cisplatin and 3 μM NVP-231. (F) 5 μM cisplatin and 10 μM NVP-231. CDDP: cisplatin.

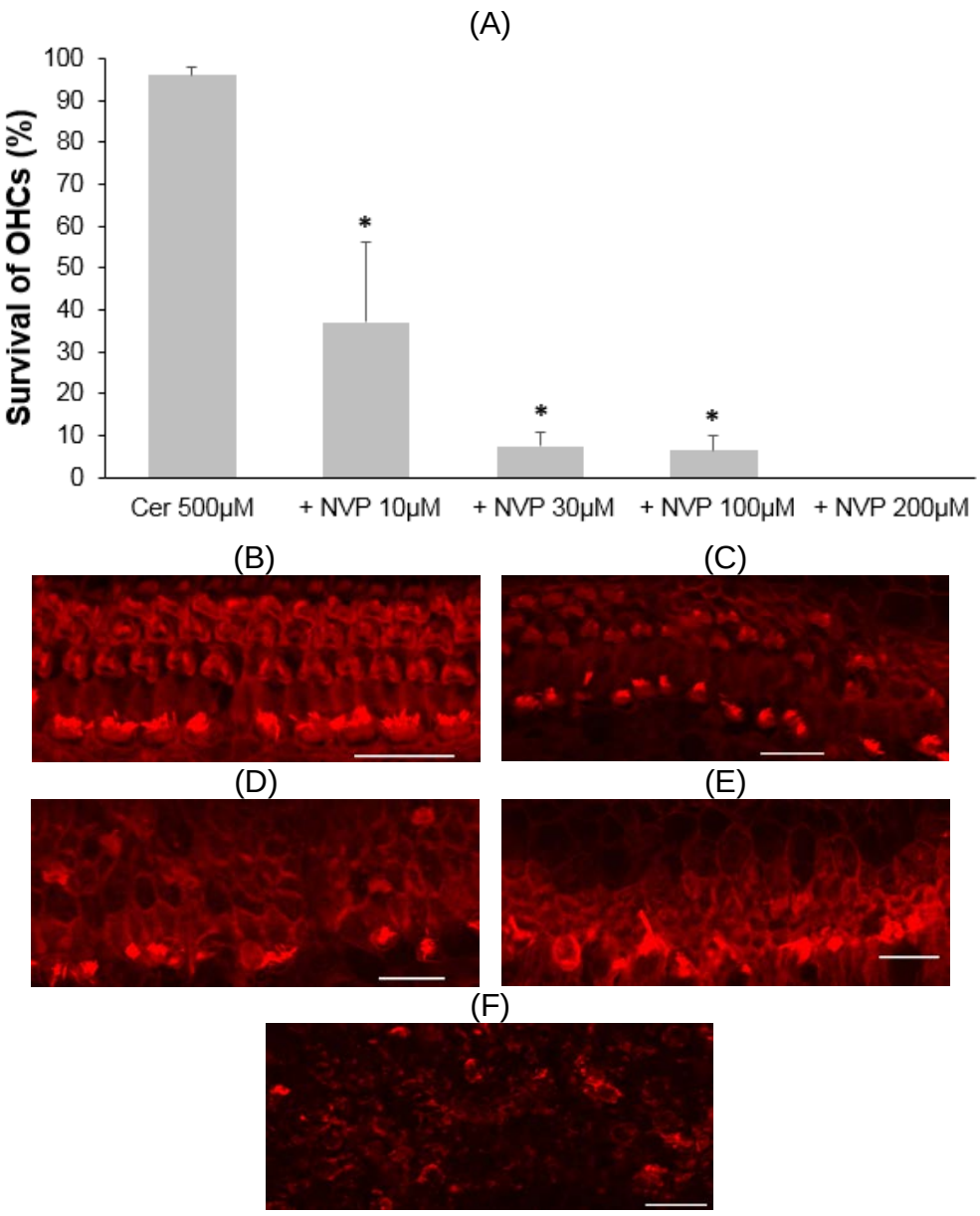


Figure 2.12. Effects of NVP-231 on cochlear hair cell treated with ceramide. (A) Quantitative analysis of cochlear OHCs. The survival of cochlear OHCs was statistically decreased when NVP-231, starting from 10 to 200 μM, was added to culture media containing 500 μM ceramide (ANOVA followed by Bonferroni post hoc test, *P = 0.000). (B – F) Representative photographs of each group (scale bar 20 μm). (B) 500 μM ceramide alone. (C) 500 μM ceramide and 10 μM NVP-231. (D) 500 μM ceramide

and 30 μM NVP-231. (E) 500 μM ceramide and 100 μM NVP-231. (F) 500 μM ceramide and 200 μM NVP-231; all explants were severely damaged. Cer: ceramide.

2.4. Discussion

It has been demonstrated that C1P participates in several vital pathophysiological functions including regulation of cell growth and survival, stimulation of DNA synthesis and cell division, and inhibition of cellular apoptosis (Gomez-Munoz, Frago et al. 1997, Carpio, Stephan et al. 1999, Gomez-Munoz 2004). Previous studies also suggested that functions of C1P might be specific to cell types. In the present study, its involvement in cisplatin-induced hair cell death was investigated. This is the first time we showed that cisplatin-induced cochlear OHC death was successfully suppressed by using C1P. Previously, C1P was acknowledged for its anti-apoptotic potential, but in different experiment conditions from ours. Gomez-Munoz laboratory showed that additional C1P treatment to culture media without macrophage colony-stimulating factor blocked the activation of caspase, prevented DNA fragmentation, and led to stability of macrophage viability (Gomez-Munoz, Kong et al. 2004). Earlier, Frago et al also recorded the effect of C1P in suppression of starvation-induced cell death (Frago, Leon et al. 1998).

In the first few hours of cisplatin exposure, cochlear hair cells enhance their defence mechanisms by inducing the upregulation of several anti-apoptotic genes that are associated with the NF-κB pathway, caspase recruitment domain family, IAP family, Bcl-2 family, and p53 signaling (Ding, Ping et al. 2009). These signaling pathways are also known to be stimulated by the PI3-K/Akt pathway. Our study revealed that activation of the PI3-K/Akt pathway was at least one of the mechanisms in which C1P promotes

cochlear hair cell survival. Previous studies pointed to sphingomyelin as the major precursor for ceramide and to sphingomyelinase as the major enzyme responsible for ceramide generation. The activity of sphingomyelinase was reportedly restrained through the activation of PI3-K/Akt pathway (Testai, Landek et al. 2004) and prevented the increase of ceramide (Noda, Yoshimura et al. 2001) as its result. Another supportive result came from an experiment using sphingomyeline assay to measure activities of sphingomyelinase, and authors also pointed out that the introduction of C1P lowered sphingomyelinase activity. Moreover, their results indicated that C1P involved direct physical interaction with sphingomyelinase as it inhibited this enzyme in cell homogenates (Gomez-Munoz, Kong et al. 2004). Additionally, the activation of ERK1/2 pathway, which is related to cell growth-survival, could be another explanation of cochlear hair cell survival in our study (Fig. 2.13). ERK1/2 are critical components of the intracellular signaling networks that regulate gene expression in response to cisplatin, and ERK1/2 activation determined cell fate in response to cisplatin (Brozovic and Osmak 2007). In fact, the induction of ERK1/2 phosphorylation was reported in experimental models of C1P with osteoblastic cells (Carpio, Stephan et al. 1999) and macrophages (Gangoiti, Granado et al. 2008).

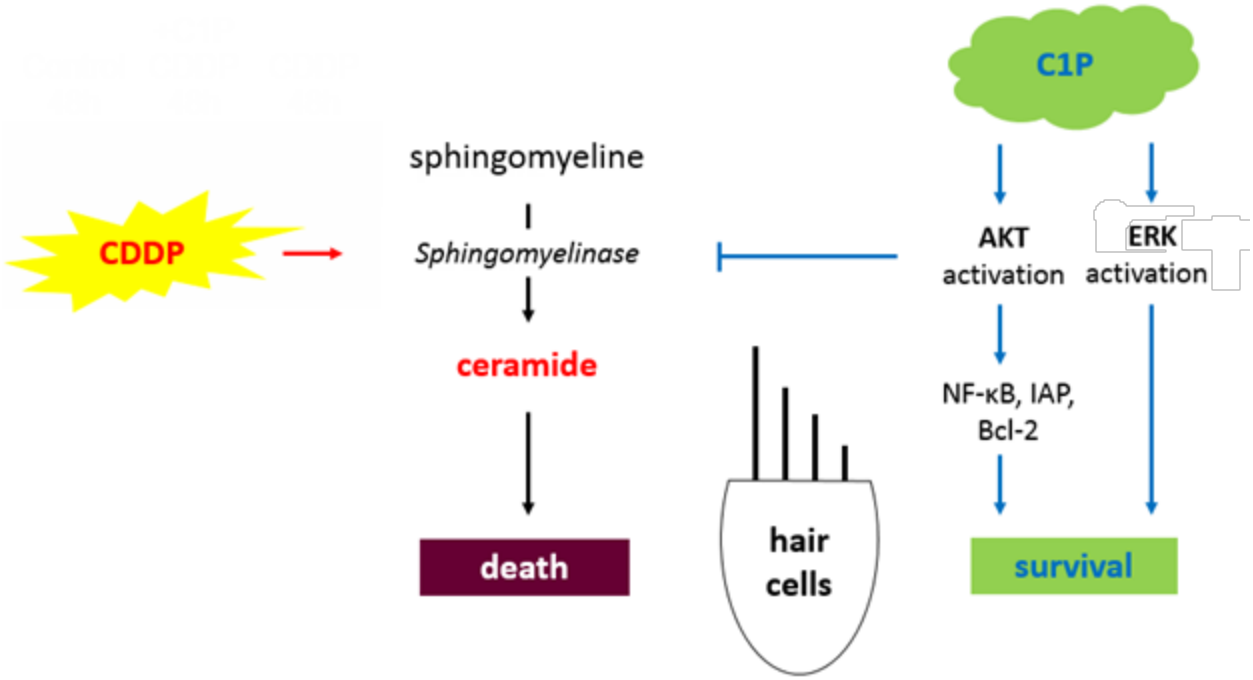


Figure 2.13. Activation of Akt and ERK1/2 pathway induced by C1P and its relation to cochlear hair cell survival

The co-administration of ceramide and cisplatin accelerated the death of cochlear OHC at high concentration in our study. It was known that cisplatin activated acid sphingomyelinase and released ceramide (Lacour, Hammann et al. 2004); therefore, it leaded to the excessive accumulation of ceramide, and could be one of explanations for the effects of this co-administration. Ceramide was known to cause the death of neuronal cells in the central and peripheral nervous systems (Cavallini, Venerando et al. 1999, Yen, Mar et al. 1999, Carpinteiro, Dumitru et al. 2008). Although precise mechanisms of ceramide induced-cell death have not fully known yet, it is currently thought that its mechanisms were related to activation of apoptotic processes (e.g., PKC, PP2A, etc.) (Ruvolo 2003), and also to inhibition of anti-apoptotic pathway (Zundel, Swiersz et al. 2000).

In the present study, ceramide alone had least effects on cochlear hair cells. In mammalian cells, CERK directly phosphorylates ceramide to synthesize C1P and is currently the only known enzyme to have this function (Sugiura, Kono et al. 2002). Therefore, we hypothesized that cochlear hair cells might have a high intrinsic activity of CERK, which rapidly phosphorylates ceramide resulting in increase of C1P synthesis; thus, cochlear hair cells were protected from the damage of high ceramide concentration. When treating cochlear hair cells with NVP-231 (a CERK inhibitor) in the presence of cisplatin or ceramide, we observed remarkable increases of apoptosis. It was the consequence of at least two processes: inhibition of ceramide phosphorylation and disinhibition of acid sphingomyelinase (Testai, Landek et al. 2004), which lead to the accumulation of ceramide and depletion of C1P. Although the measurement of C1P could not be archived due to its scantiness in cochlear explants, these results confirmed our hypothesis above and explained partly the central role of C1P in governing cochlear hair cell fate.

Taken together our results suggest the importance of the balance between ceramide and C1P. On one hand, ceramides triggers apoptosis via several pathways including blocking the PI3-K/Akt; on the other hand, C1P inhibits this process by activating the PI3-K/Akt pathway and increasing phosphorylation of ERK1/2. In short, our present study suggests that at least the balance of ceramide and C1P regulates cochlear hair cell fate, and any circumstances that alter this balance (e.g., a CERK inhibitor) will affect the survival of cochlear hair cell (Fig. 2.14).

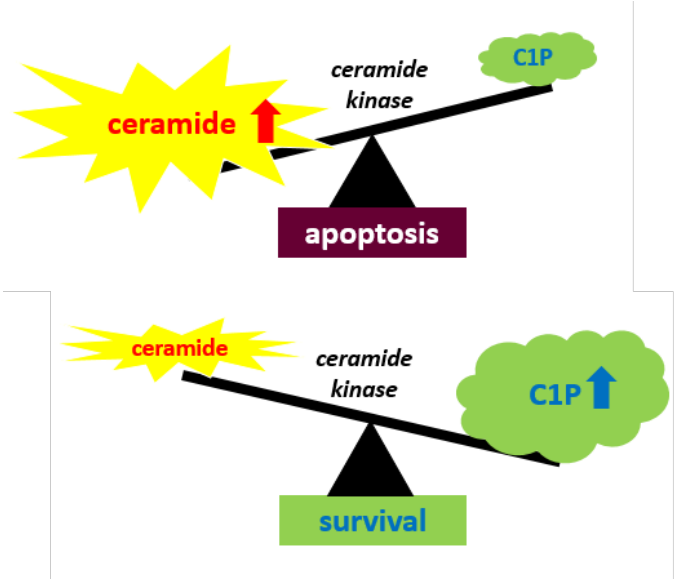


Figure 2.14. Outer hair cell fate and ceramide – C1P balance

Since the discovery of CERK over 10 years ago, some evidences showed that inhibition of CERK might enhance the efficacy of anti-cancer therapies. Firstly, significantly high expression of CERK was identified in estrogen-receptor-negative breast cancer tumors (Ruckhaberle, Karn et al. 2009) and hepatoma cell lines (Hsieh, Hsu et al. 2009). Secondly, when the activity of CERK is abolished, it greatly increases the susceptibility of tumor cell lines to low-dose UV irradiation (Hsieh, Hsu et al. 2009). Furthermore, it is suggested that the inhibition of CERK will overcome the issue of chemotherapeutic resistance by rising ceramide levels in combination of chemotherapies (Ogretmen and Hannun 2004). However, the inhibition of CERK needs to be approached with great caution, because of a possibility of an unbalance between ceramide and C1P. The elevation of ceramide level would possibly render other normal cells more vulnerable to the treatment – particularly cochlear hair cells in cisplatin ototoxicity, as the findings of our study.

2.5. Conclusion

Relied on published results, some authors recommended that effects of C1P might be cell type-specific, and this is the first time we provides evidences that C1P regulates cochlear hair cell survival in cisplatin ototoxicity through the PI3-K/Akt and ERK1/2 pathway. In addition, the data pointed out that this survival follows the balance of ceramide and C1P. Cisplatin and CERK inhibitor are some of the factors shifting this balance to the ceramide side. While the former was known to increase ceramide level by activating its generation, the latter inhibits the phosphorylation process leading to the increase of ceramide accumulation. In contrast, this balance will move to C1P side when there is an endogenous or exogenous acceleration of C1P.

Chapter 3. The role of peroxiredoxin I in cisplatin-induced ototoxicity

3.1. Introduction

Peroxiredoxins (Prxs) are a family of small antioxidative proteins from 22 to 27 kDa (Kim, Kim et al. 1988, Chae, Kim et al. 1993). To date, 6 Prx subtypes have been identified in mammals (Knoops, Clippe et al. 1999, Seo, Kang et al. 2000), and can be divided into three major subclasses: typical 2-cysteine Prxs (Prx 1 to 4), atypical 2-cysteine Prx (Prx 5) and 1-cysteine Prx (Prx 6).

Prxs have been shown to be related to diverse cellular roles (Rhee and Woo 2011), among which scavenging reactive oxygen species (ROS) via reducing equivalents has generated a great interest. Particularly, Prxs catalyze the reduction in hydrogen peroxide, alkyl hydroperoxides, and peroxynitrite using reducing equivalents provided by thioredoxin (Hofmann, Hecht et al. 2002). Among the 6 subtypes, Prx I is found mainly in the cytosol and is ubiquitously expressed in various tissues (Uwayama, Hirayama et al. 2006); for example, it is highly expressed in the liver, kidney, and small intestine (Ishii, Yamada et al. 1993, Ishii, Itoh et al. 2000). The major roles of the Prxs are related to cellular protection against oxidative stress, to the modulation of intracellular signaling cascades using hydrogen peroxide as a second messenger, and to the regulation of cell proliferation. Expression of the Prx I gene is upregulated by various stress agents and is considered to be a cellular recovery response (Ishii, Yamada et al. 1993, Chang, Jeong et al. 2002). In a previous study conducted by our colleagues (Uwayama, Hirayama et al. 2006), the liver and kidney of the Prx I-deficient mouse were significantly sensitive to

increases in ROS, and we therefore hypothesized that a similar phenomenon may also be present in the mouse cochlea.

As we described in a previous chapter, cisplatin is well known as a chemotherapeutic agent against malignant tumors (Boulikas and Vougiouka 2004). Regardless of its efficiency, its clinical application is often limited by the associated side effects. Of these, ototoxicity is one of the most serious concerns because of the risk of permanent hearing loss associated with cochlear damage. The ototoxic mechanisms of cisplatin have gradually been identified, and oxidative stress has been implied as one of the main pathophysiologies (Rybak, Whitworth et al. 2007, Goncalves, Silveira et al. 2013). Animal research indicates that reduced auditory acuity is partially mediated by free radical generation and antioxidant inhibition (Evans and Halliwell 1999). Other in vitro experiments showed that cisplatin raises ROS levels and depletes the intracellular concentration of glutathione (Hanigan and Devarajan 2003), and lead to cell apoptosis (Ueda, Kaushal et al. 2000). It is well known that antioxidative enzymes (eg, superoxide dismutase and glutathione peroxidase) are essential to scavenge the ROS involved in the oxidative stress induced by cisplatin to protect cells from further damage. Therefore, various kinds of antioxidant therapies have been tried to ameliorate the ototoxicity of cisplatin (Li, Frenz et al. 2001, Teranishi and Nakashima 2003). However, less is known about the involvement of Prxs in the cochlea.

In the present study, we examined the expression of Prx subtypes in the mouse cochlea and showed how Prx I participates in protecting the cochlea against cisplatin through comparing effects of cisplatin on wildtype mice and Prx I-deficient mice.

3.2. Method

3.2.1. Animals and cochlear explants

C57BL/6J mice and Prx I-deficient mice from postnatal days 3 to 5 were used. Prx I-deficient mice were generated as described previously (Uwayama, Hirayama et al. 2006). For the cochlear explants, we used basal turns of the organ of Corti dissected and cultured according to the methods of Van de Water and Ruben (Van de Water and Ruben 1974) and Sobkowicz et al (Sobkowicz, Loftus et al. 1993). All animal procedures were conducted according to the guidelines of the Laboratory Animal Research Center of the University of Tsukuba.

3.2.2. Cisplatin treatment

Cochlear explants from both wildtype and Prx I-deficient mice were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 25 mM HEPES, and 30 U/mL penicillin. The cultures were incubated at 37^{o}C with 5% CO_2 at 95% humidity. The cochlear explants were initially stabilized in the above-described medium overnight (8 – 12 hours) before transferred to a new medium plus 2, 5, or 10-μM cisplatin for 48 hours.

3.2.3. Cytochemistry

At the end of the culture process, each experimental group, which consisted of 11 to 12 cochlear explants, was fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 minutes and then permeabilized with 5% Triton X-100 (Sigma, St. Louis, MO, USA) in PBS with 10% fetal bovine serum (FBS) for 10 minutes. The explants

were stained with phalloidin with a conjugated Alexa Fluor probe (1:100; Molecular Probes, Carlsbad, CA, USA) at room temperature for 1 hour (Tabuchi, Pak et al. 2007). Phalloidin is a specific marker for cellular F-actin and labels stereociliary arrays and the cuticular plates of hair cells. Cochlear explants were transferred to a glass slide and examined with a FluoView F10i confocal microscope (Olympus, Center Valley, PA, USA). Hair cells were characterized as missing if no stereocilia or no cuticular plates were observed by phalloidin staining. Quantitative results were obtained by evaluating 30 OHCs associated with 10 inner hair cells in a given microscopic field. The average of three randomly separated counts was used to represent each tissue culture. The residue of cochlear hair cells was expressed as a percentage and the results of each group were compared (Tabuchi, Pak et al. 2007).

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

After a postnatal pup was decapitated, cochlea was dissected, ear capsule were removed, and three main parts of cochlea were isolated, including organ of Corti, lateral wall and modiolus. Total ribonucleic acids (RNA) of the organ of Corti, the modiolus, the lateral wall were extracted using Qiagen RNeasy mini kit (Qiagen Inc., Valencia, CA, USA). To avoid any DNA contaminations, DNase I (Qiagen Inc., Valencia, CA, USA) was added to RNA extraction column following the instruction of the supplier. 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 were detected by conventional reverse transcription polymerase chain reaction with Taq

polymerase enzyme (Takara, Shiga, Japan) by Veriti Thermal Cycler (Life Technologies, Grand Island, NY, USA). Prx primer sequences were summarized in table 3.1 and glyceraldehyde-3-phoshate dehydrogenase (GAPDH) was used as an internal control. All gene sequences were accessed from GenBank®.

Abbreviation	Names	Accession number	Forward primer / Reverse primer	Amplicon size (bp)
Prx1	Mus musculus peroxiredoxin 1	NM_011034.4	ACGACTAGTCCAGGCCTTCC GGCAGAAAAATGGTCCAGTG	151
Prx2	Mus musculus peroxiredoxin 2	NM_011563.5	CTCCTGACTTCACGGCCACA GAAGTCCTCAGCATGGTCGCTAA	161
Prx3	Mus musculus peroxiredoxin 3	NM_007452.2	GGTGCTTTTCTTCTACCCTTTGG CACAGTTTACATCATGAAATTCATTGG	98
Prx4	Mus musculus peroxiredoxin 4	NM_016764.4	TGGACGAGACACTGCGTTTG CTGGATCTGGGATTATTGTTTCACTAC	108
Prx5	Mus musculus peroxiredoxin 5	NM_012021.2	GGAAGGCGACAGACTTATTATTGG CTCCACGTTCAGTGCCTTCAC	116
Prx6	Mus musculus peroxiredoxin 6	NM_007453.3	GCCAAGAGGAATGTTAAGTTGATTG GTTTCACCATTGTAAGCATTGATGTC	95
Actb	Mus musculus actin, beta	NM_007393	CATTGCTGACAGGATGCAGAAGG TGCTGGAAGGTGGACAGTGAGG	138
GAPDH	Mus musculus GAPDH	NM_001289726.1	TGTGTCCGTCGTGGATCTGA TTGCTGTTGAAGTCGCAGGAG	150

Table 3.1. Primer sequences for real-time PCR

3.2.5. Real-time PCR assay for mRNA

The cochlear explants of C57BL/6J mice were cultured in different conditions, including a normal culture medium or a culture medium plus 5-μM cisplatin for 4, 8, 24,

or 48 hours. Total RNAs of the cochlear explants were extracted as described above. Total RNA was quantitated on a NanoDrop 2000 UV-Vis spectrophotometer (Thermo Scientific, Wilmington, DE, USA), and equal amounts of total RNA (140 ng) were used for all real-time PCR assays. mRNA was primed with random hexamers, and complementary DNA (cDNA) was synthesized from mRNA by using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The cDNA was amplified by using SsoFast EvaGreen Supermix with Low ROX (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. The primers used for the real-time PCR are listed in Table 3.1; beta actin was used as the internal control. The final mixture of the PCR reaction (total 20 µL) contained 1 µL of cDNA, 0.4 µL of 10 µmol/L forward primer, 0.4 µL of 10 µmol/L reverse primer, 10 µL of Bio-Rad PCR buffer, and 9.8 µL of DEPC-treated water. The PCR reaction was repeated in duplicate (2 wells of a 96-well plate), and the whole assay was repeated in triplicate. The change in the expression of each gene was measured by using the ABI 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) with the optimized PCR protocol for the Bio-Rad PCR buffer. The relative quantification value of the expression of the gene of interest was calculated from the raw threshold cycle (Ct). The Ct values of beta actin were subtracted from the Ct values of the genes of interest to calculate the ΔCt. The ΔCt values from each experimental group were averaged. Values from the equation $2^{(-\Delta Ct)}$ were used to compare the different expressions of the genes of interest, and the data were presented as the means ± SDs of the triplicate assays.

3.2.6. Immunofluorescence

Immunofluorescence staining was performed to detect expression of Prx I in mouse cochlea. For sectioned immunofluorescence staining, both cochleae of postnatal-day-3-to-5 and adult wildtype mice were used. Collecting method of cochlea in postnatal mice was described above, however adult mice had to anesthetized deeply with pentobarbital sodium before dissection. Cochlea with intact capsule was fixed with 4% paraformaldehyde, decalcified with EDTA, and then embedded in paraffin. The cochleae were cut into 4-μm-thick midmodiolar sections. After deparaffinized, sections was immersed in citrate buffer and heated in a microwave before proceeding other immunostaining steps, which were similar to whole-mount immunostaining.

For whole-mount immunofluorescence staining, cochlear explants of wildtype mice were fixed with 4% paraformaldehyde for 10 minutes at room temperature. After washed 3 times with PBS, the cochlear explants were permeabilized in 3% Triton X-100 solution for 30 minutes at room temperature and then washed again 3 times with PBS before blocked with 10% normal goat serum for 30 minutes at room temperature. Next, the blocking solution was removed, and the cochlear explants were rinsed with PBS before incubated with polyclonal rabbit anti-Prx I (1:50; #15816-1-AP; ProteinTech Group, Chicago, IL, USA) for 72 hours at 4^{o}C. After cochlear explants were washed 3 times with PBS, the goat anti-rabbit secondary antibody conjugated to an Alexa Fluor 488 probe (1:200; #4412; Cell Signaling Technology, Beverly, MA, USA) was applied overnight at 4^{o}C in darkness. For fluorescence visualization of the hair cell structure, the cochlear explants were incubated with rhodamine phalloidin (1:100; Invitrogen) at room temperature for 60 minutes. The cochlear explants were mounted on slides with antifade

solution and examined using a FluoView F10i confocal microscope (Olympus). The same procedure was repeated more than 3 times to confirm the results.

3.2.7. Assessment of spiral ligament fibrocytes

Cochleae of postnatal-day-3-to-5 wildtype and Prx I-deficient mice were collected. After the bony capsule was gently removed, the cochlear samples were cultured for 48 hours in media containing 5, 10, 20, 50, or 100-μM cisplatin. The cochlear samples were fixed with 4% paraformaldehyde and then embedded in paraffin. The cochlear samples were cut into 4-μm-thick midmodiolar sections. After the sections were stained with hematoxylin and eosin, images of the sections at the same magnification (x20) were collected by using a BZ-X710 microscope (Keyence, Itasca, IL, USA). The quantities of the spiral ligament fibrocytes of lateral wall were counted from 3 random circular areas (r = 15 μm) in 1 section, and the same procedure was performed for 3 different sections of 1 cochlear sample.

3.2.8. Data analysis

All data were expressed as means ± SDs. Statistical analysis was performed using unpaired *t* tests or one-way analysis of variance (ANOVA) with Bonferroni post hoc tests, as required (SPSS 20). Probability values less than 0.05 were considered significant.

3.3. Results

3.3.1. Expression levels of the Prx subtypes

To ensure the mRNA quality and expression, RT-PCR analysis was conducted. The data showed that, mRNA of 6 subtypes were presented in organ of Corti, lateral wall and modiolus (Fig. 3.1). Brain tissues were used as a positive control in this study due to its well-known expression of 6 Prx subtypes. Next, expression of the 6 Prx subtypes in the cochlear explants was observed by real-time PCR at 4, 8, 24, and 48 hours following 5-μM cisplatin treatment. After 48 hours of cisplatin exposure, subtypes I, IV, and V showed significant upregulations (Fig. 3.2; ANOVA with Bonferroni test; Prx I, p = 0.00001; Prx IV, p = 0.001; Prx V, p = 0.002), while subtypes II and VI showed significant downregulations (Fig. 3.2; ANOVA with Bonferroni test; Prx II, p = 0.004; Prx VI, p = 0.0001). Subtype III did not show any statistically different change. Of the 6 subtypes, Prx I and II were the two most abundant in terms of mRNA expression.

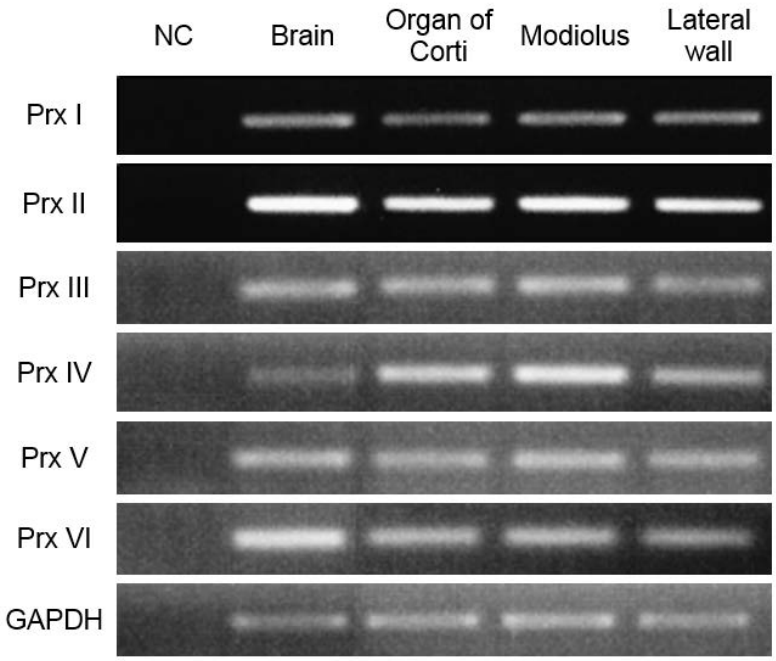
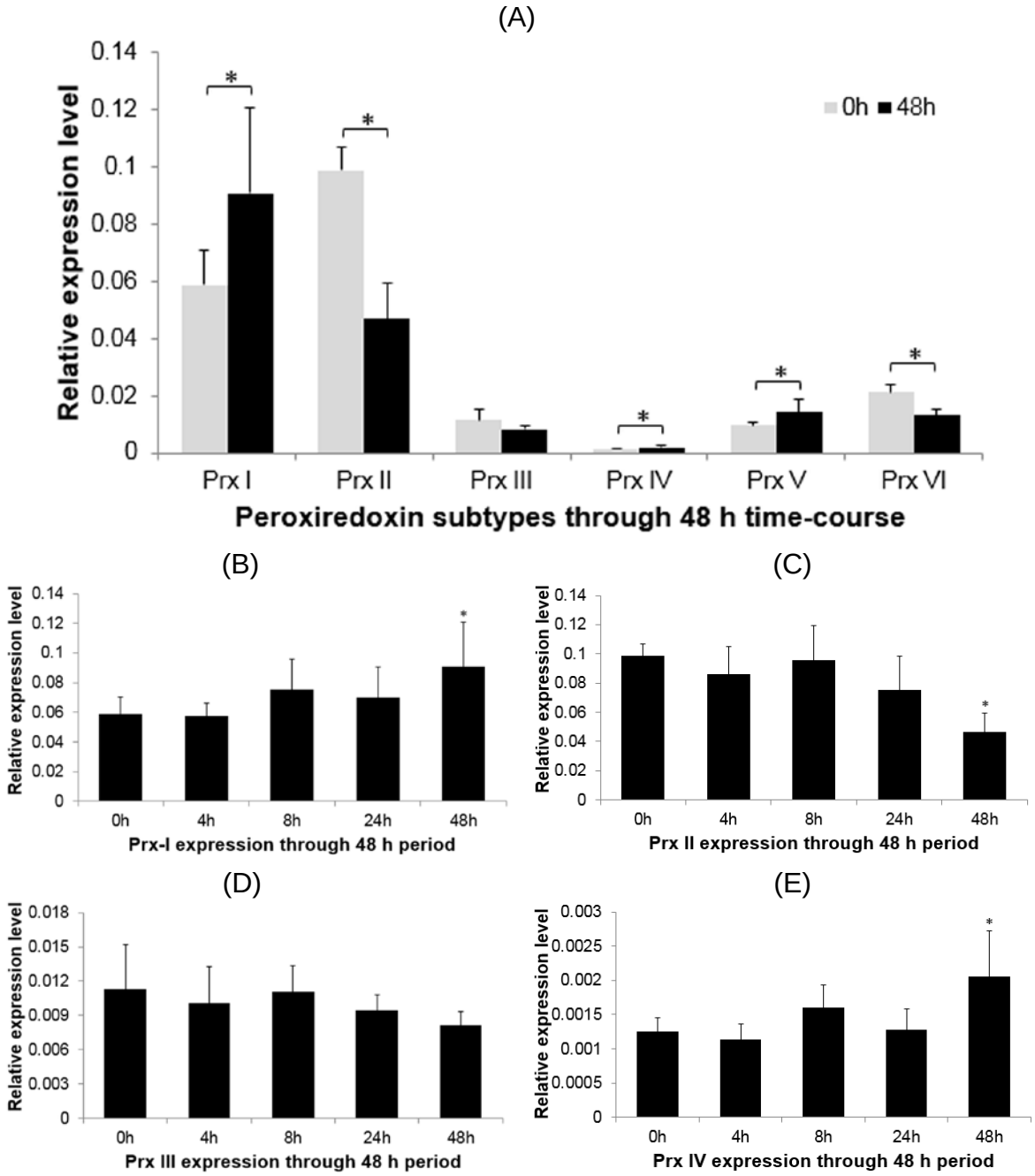


Figure 3.1. Prx subtype expression in cochleae of wildtype mice.

RT-PCR analysis demonstrated that 6 subtypes of Prxs were expressed in the organ of Corti, modiolus and lateral wall. GAPDH primers served as the cDNA loading control. The brain tissues were used as a positive control. NC: negative control.



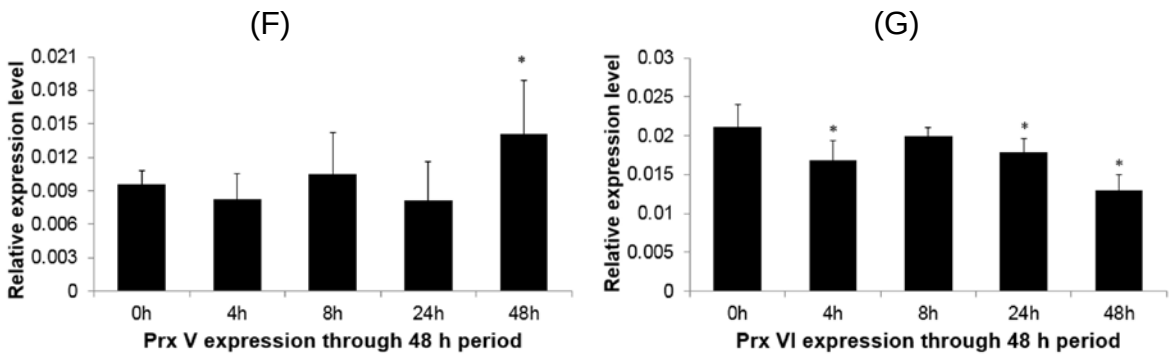


Figure 3.2. The cisplatin-induced regulation of Prxs in cochleae of wildtype mice. (A) Different expressions of Prx subtypes after 48 hours of cisplatin exposure. The expression of subtypes I, IV and V increased significantly (ANOVA followed by Bonferroni test; $*p < 0.05$), while the expression of subtypes II and VI decreased significantly (ANOVA followed by Bonferroni test; $*p < 0.05$). Subtype III showed no significant difference. (B – G) The time course of Prx subtype expression induced by cisplatin through 48 hours.

3.3.2. Immunofluorescence signals of Prx I in normal cochlear explants

The location of Prx I proteins in the organ of Corti was determined by immunolabeling with polyclonal rabbit anti-Prx I antibodies (Fig. 3.3). Signals for the Prx I proteins were observed universally in the cochlea, including in the organ of Corti, the lateral wall, and the spiral ganglion. In the highly magnified view, Prx I was detected in the cochlear IHCs and OHCs and in the supporting cells. The Prx I signals in the cochlear IHCs and OHCs were weaker than those in the supporting cells in the whole-mount immunofluorescence staining.

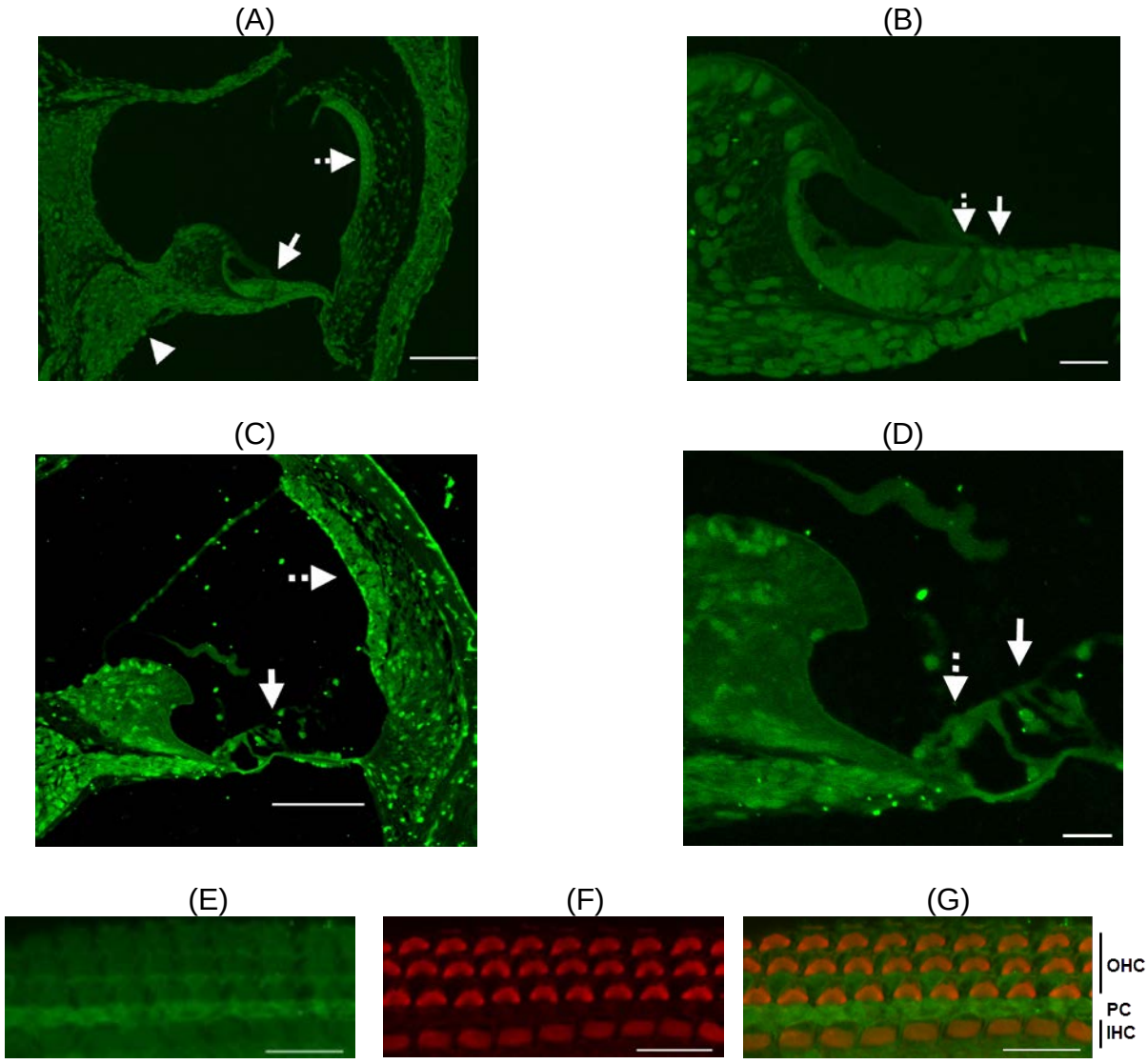
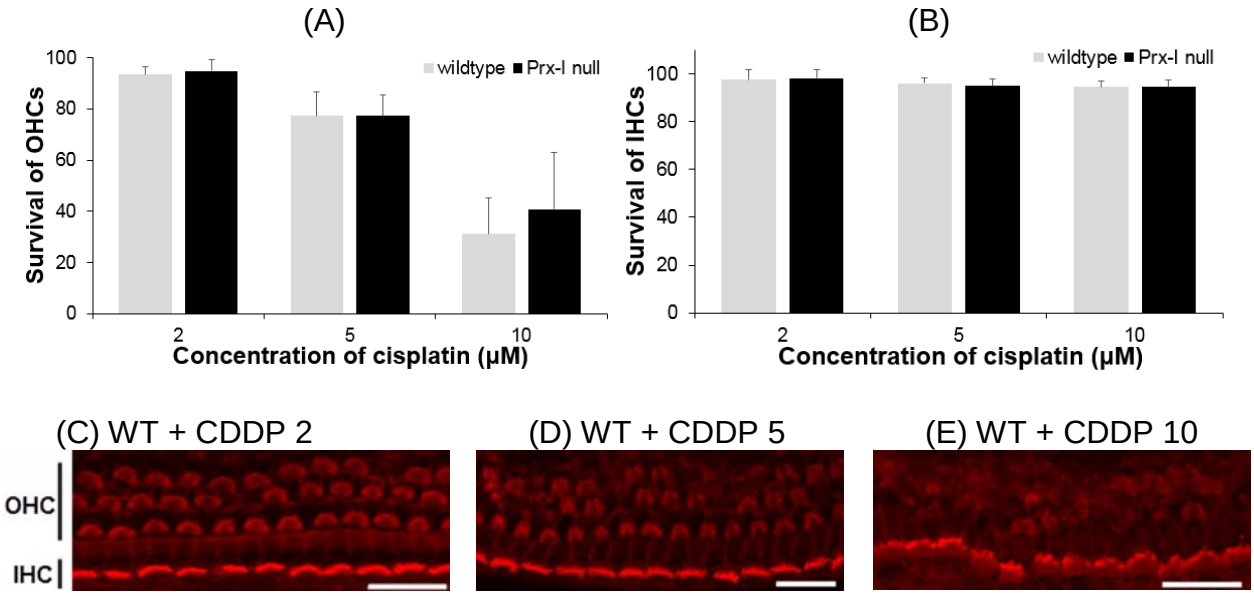


Figure 3.3. Immunofluorescence of Prx I in wildtype cochlear explants. Representative photographs of mid-modiolar cross-section in postnatal cochlea (A – B) and adult cochlea (C – D); (scale bar 100 μm in A, C, and 20 μm in B, D). Prx I was expressed ubiquitously in organ of Corti (solid arrow), lateral wall (dashed arrow) and spiral ganglion (head arrow). In magnified view of the organ of Corti, Prx I was expressed in the cochlear IHCs (dashed arrow), OHCs (solid arrow), and supporting cells around and beneath hair cells. (E – G) Representative photographs of whole-

mount immunofluorescence staining (scale bar 20 μm). (E) Prx I was immunolabeled in green color. (F) Rhodamine Phalloidin staining (*red*) for cochlear hair cell visualization. (G): Merged image of Prx I and cochlear hair cells. Cochlear IHCs and OHCs showed weaker signals than supporting cells. OHC: outer hair cells, IHC: inner hair cells, PC: pillar cells.

3.3.3. Cisplatin-induced cochlear hair cell loss in Prx I-deficient mice

Cochlear explants of both wildtype and Prx I-deficient mice were treated with cisplatin at concentrations of 2, 5, and 10 μM for 48 hours. After the cisplatin treatment, the cochlear hair cells were counted. The statistical comparisons between the groups revealed no significant differences in the numbers of the residual OHCs (Fig. 3.4A; unpaired *t* test; 2-μM cisplatin, $p = 0.26$; 5-μM cisplatin, $p = 0.48$; 10-μM cisplatin, $p = 0.21$). In addition, no differences were found in comparison of residual inner hair cells (Fig. 3.4B; unpaired *t* test; 2-μM cisplatin, $p = 0.35$; 5-μM cisplatin, $p = 0.25$; 10-μM cisplatin, $p = 0.5$).



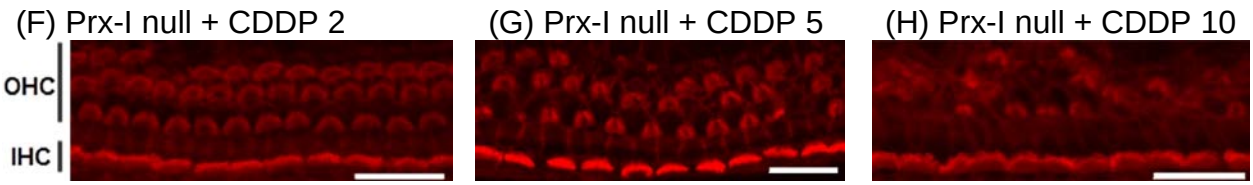


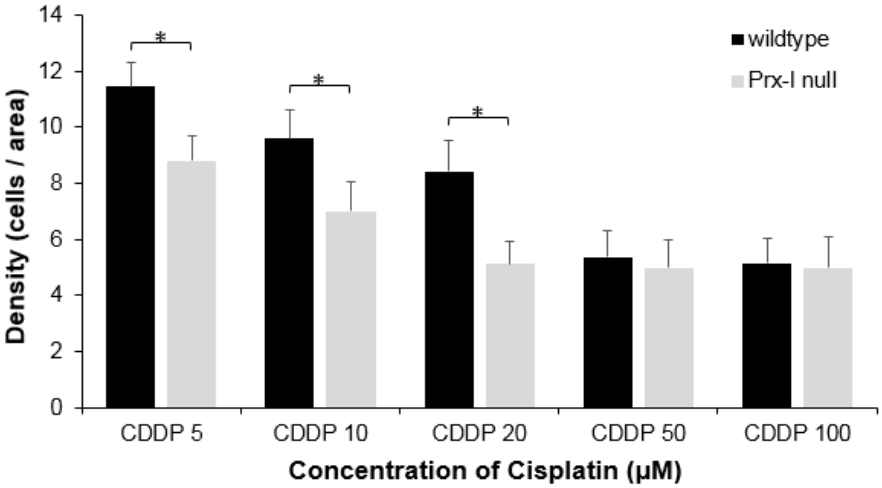
Figure 3.4. Cisplatin-induced cochlear hair cell loss in wildtype and Prx I-deficient mice.

(A – B): Quantitative analysis of cochlear IHCs and OHCs between wildtype and Prx I-deficient mice. There was no statistical difference between the groups (unpaired t-test; $p > 0.05$). (B – G) Representative photographs of cochlear hair cells exposed to cisplatin in each group (scale bar 20 μm). 2 (C), 5 (D) and 10 (E) μM cisplatin in wildtype mice. 2 (F), 5 (G) and 10 (H) μM cisplatin in Prx I-deficient mice. OHC: outer hair cells; IHC: inner hair cells; WT: wildtype; CDDP: cisplatin

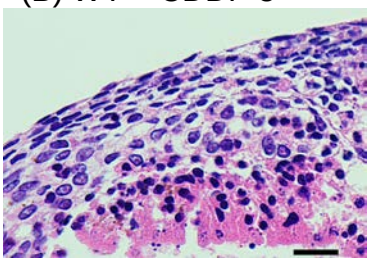
3.3.4. Vulnerability of spiral ligament fibrocytes to cisplatin in Prx I-deficient mice

Next, the density of the spiral ligament fibrocytes in the midmodiolar sections of postnatal-day-3-to-5 wildtype and Prx I-deficient mice were calculated. To ensure unified data collection, the upper basal turns of the cochlea were selected (Fig. 3.5). Cisplatin decreased the fibrocytes in both the wildtype and the Prx I-deficient mice. Quantitative analysis of the fibrocytes revealed that the number of residual fibrocytes was significantly more decreased in the Prx I-deficient mice than in the wildtype mice after 5, 10 and 20-μM cisplatin exposure (unpaired *t* test; $p = 0.00005$; $p = 0.002$; $p = 0.001$ respectively). Meanwhile, at higher concentrations of cisplatin, cisplatin damaged the fibrocytes severely and no statistical differences were found between the groups (unpaired *t* test; 50-μM cisplatin, $p = 0.239$; 100-μM cisplatin, $p = 0.791$)

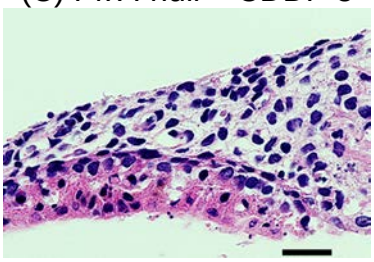
(A)



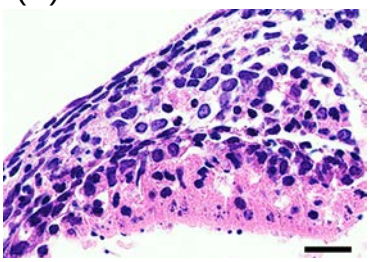
(B) WT + CDDP 5



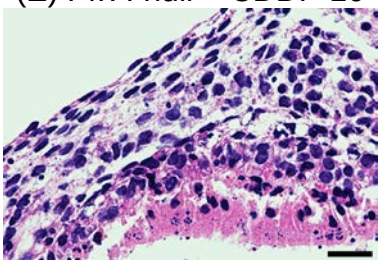
(C) Prx-I null + CDDP 5



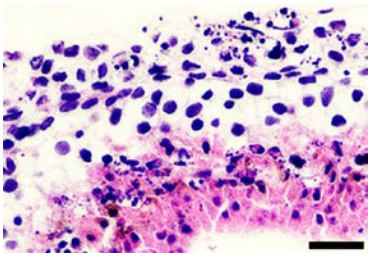
(D) WT + CDDP 10



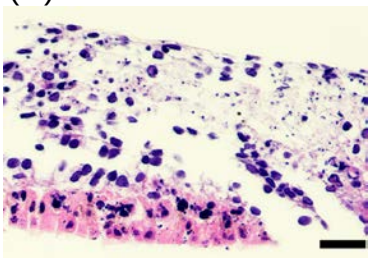
(E) Prx-I null + CDDP 10



(F) WT + CDDP 20



(G) Prx-I null + CDDP 20



(H) WT + CDDP 50

(I) Prx-I null + CDDP 50

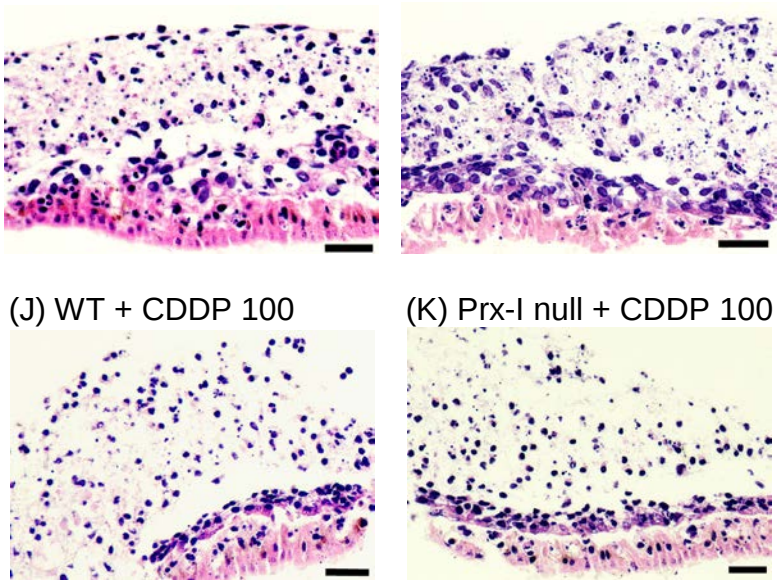


Figure 3.5. Vulnerability of spiral ligament fibrocytes in Prx I-deficient mice. (A) Density of spiral ligament fibrocytes in lateral wall between wildtype and Prx I-deficient mice. The densities were calculated by number of cells per a circular area (r = 15 μm). Prx I-deficient mice had significantly lower density at 5, 10 and 20 μM cisplatin (unpaired t-test; $*p < 0.05$); meanwhile, no differences were found at 50 and 100-μM (unpaired t-test; $p > 0.05$). (B – K) Representative photographs of spiral ligament fibrocytes exposed to cisplatin in each group (scale bar 20 μm). 5 (B), 10 (D), 20 (F), 50 (H) and 100-μM (J) cisplatin in wildtype mice. 5 (C), 10 (E), 20 (G), 50 (I) and 100-μM (K) cisplatin in Prx I-deficient mice. WT: wildtype; CDDP: cisplatin

3.4. Discussion

The antioxidative function of Prx has been shown to be related to the pathologies of diseases in various organ systems; however, little is known about its role in the otologic system.

In this present study, we first showed the mRNA expression of all 6 subtypes of Prxs in the mouse organ of Corti and that Prx I and II were the 2 most abundant subtypes. Godoy et al used immunohistochemistry to build a redox atlas of the mouse and showed that several mouse organs expressed all 6 Prx subtypes (Godoy, Funke et al. 2011). However, the predominance of each Prx subtype differed from organ to organ. Although Prx I was wildly expressed in most of the organs and tissues, Prx I is not necessarily always the most abundant subtype. For example, Prx I was strongly expressed in the central nervous system whereas Prx II was dominantly expressed in the heart, skeleton, and uterus (Immenschuh and Baumgart-Vogt 2005, Godoy, Funke et al. 2011). Therefore, the higher expression of Prx II than of Prx I in our study may be a characteristic Prx expression pattern in the organ of Corti. Besides, more important conclusions of both the Godoy and the Immenschuh studies (Immenschuh and Baumgart-Vogt 2005, Godoy, Funke et al. 2011) were that different cell types of a same organ had different expressions of the 6 Prx subtypes.

When the organ of Corti was exposed to cisplatin, the mRNAs of Prx I, IV, and V were upregulated. Similarly, in the study of Cho et al, mRNAs of Prx I and V were also upregulated in cisplatin-induced hepatotoxicity (Cho, Singh et al. 2012). Furthermore, experiments with other cell lines showed that the upregulation of Prx I was not limited to cisplatin but extended to various other oxidative stimuli (Ishii, Yamada et al. 1993, Bast, Wolf et al. 2002, Kim, Manevich et al. 2003). On the other hand, we also recognized the mRNA downregulation of Prx II and VI in the organ of Corti after cisplatin exposure. Dahlan et al (Dahlan, Karsani et al. 2012) showed that Prx II and VI of lymphocytes were downregulated in response to H2O2. Prx II is expressed exclusive in neurons (Sarafian,

Verity et al. 1999), and its abundance in cochlea may contribute to protect neuronal structures against oxidative stimuli. Although the exact molecular mechanism of Prx II down-regulation remains unclear, its down-regulation was reported to be produced by overoxidation induced by stimuli such as cisplatin or H2O2 (Dahlan, Karsani et al. 2012). Prx VI was also downregulated in response to stress stimuli in hepatocytes (Roede, Stewart et al. 2008) or lens epithelial cells (Pak, Kim et al. 2006). Therefore, the reaction of an individual Prx subtype to harmful stimuli may be conserved from cell to cell.

In this study we used Prx I-deficient mice to understand role of Prx I in cisplatin ototoxicity. The expression of Prx I was confirmed by RT-PCR results above and immunohistochemistry findings of whole-mount tissues and sectioned tissues, which shows its ubiquitous expression in cochlea. Cohclear hair cells and lateral wall of cochlea were at least two major targets of cisplatin (van Ruijven, de Groot et al. 2005, van Ruijven, de Groot et al. 2005) and they were selected to examine. We found that spiral ligament fibrocytes of the lateral wall significantly decreased after cisplatin exposure in Prx I-deficient mice; meanwhile, no statistical difference was found in outer and inner hair cell loss. Our finding for spiral ligament fibrocytes is supported by the study of Ma et al, who showed that fibroblasts, also derived from the embryos of Prx I-deficient mice, were vulnerable to cisplatin-induced apoptosis (Ma, Warabi et al. 2009), and ultraviolet radiation (Ito, Kimura et al. 2013). At least one possible explanation for the difference in the results between cochlear hair cells and spiral ligament fibrocytes in cisplatin ototoxicity is that different origins of these cells leads to different roles for the Prx subtypes. As mentioned above, Godoy study showed that different cell types have different expressions of Prx subtypes, although these cell types involve a same organ,

and this finding was proved in most of organs (Godoy, Funke et al. 2011). Similarly, in the review of Immenschuh, the author collected information from many studies and concluded that individual Prxs were found to have a characteristic cell-type and organ-specific expression pattern (Immenschuh and Baumgart-Vogt 2005). One of the examples is that Prx I showed moderate expression level in proximal tubular cells, whereas Prx II, III and V were strongly expressed in distal tubules (Oberley, Verwiebe et al. 2001); or in lung, bronchial epithelium, alveolar epithelium and alveolar macrophages were showed different expression patterns of Prx subtypes (Kinnula, Lehtonen et al. 2002). An additional explanation is that Prx I may possibly not be the predominant subtype in cochlear hair cells. Western blotting analysis using a cell line of cochlear hair cell (UB/OC-1) showed that Prx III was the most prominent subtype but not Prx I (Choi, Park et al. 2008). Moreover, when Prx III siRNA was delivered to the inner ear, apoptosis of the cochlear OHCs significantly increased in response to noise trauma or aminoglycoside treatment (Chen, Zheng et al. 2013).

Between cochlear OHCs and lateral wall, although there was no critical evidence of where cisplatin affects firstly, the fact that infusion of cisplatin induced the reduction of endocochlear potential (van Ruijven, de Groot et al. 2005) suggests that cochlear lateral wall might be the earlier target of cisplatin. Spiral ligament fibrocytes of the lateral wall have a vital function in recycling K+, providing K+ for the endolymph and thereby keeping the endolymph ionic concentration in balance (Zdebik, Wangemann et al. 2009). In addition, spiral ligament fibrocytes are closely involved with blood-cochlear barrier functioning in preventing ototoxic drugs (Juhn, Hunter et al. 2001), and the degradation of these fibrocytes showed a strong connection to hearing loss (Hequembourg and

Liberman 2001). In the present study, the vulnerability of the spiral ligament fibrocytes of Prx I-deficient mice indicates that Prx I may be involved in defense of the cochlea against cisplatin toxicity via fibrocyte protection. The expression of Prx I in this cell type assists the cleanup of reactive oxygen species and restrains the access of cisplatin to endolymph, which would destroy cochlear hair cells later. In vivo experiments also advocate this point since systemic administration of cisplatin at high dose showed little evidence of cochlear pathology while significant nephrotoxicity occurred; in contrast, applying cisplatin to mouse round windows or infusing it into middle ear space showed a considerable cochlear hair cell loss (He, Yin et al. 2009).

3.5. Conclusion

The present study provides evidence that mRNAs of all 6 Prx subtypes are expressed in the organ of Corti and showed their changings following cisplatin exposure. We hope this data could provide useful information for further inner ear research of Prx which are limited at the moment. Besides, Prx I showed its importance to spiral ligament fibrocytes of the lateral wall in cisplatin ototoxicity.

Chapter 4. Conclusions and perspectives

4.1. Ceramide – 1 – phosphate

Our results in chapter 2 provided further understandings of C1P and ceramide metabolism in cisplatin-induced cochlear hair cell death. Because C1P and ceramide could be interchangeable intracellularly, regulation of the enzymes involved in the metabolism of ceramides and C1P is vital for controlling the overall signal which is finally transmitted in cochlear hair cell. As well, because ceramide and C1P have opposing effects, these findings add a further understanding of the metabolic interrelationship of these metabolites particularly in cochlear hair cells, and suggest that alterations in the balance of the intracellular levels of ceramides and C1P can affect cochlear hair cell survival. Together with previous studies in our laboratory related to sphingolipid, we are gradually drawing a full picture of sphingolipid metabolites in ototoxicity which help us to partly understand toxic mechanisms of not only cisplatin but also gentamycin. With this understanding, we hope that it may help to find a cure or a prevention for patients who need to use platinum-based drugs.

Up to now, it is not known whether C1P is present in plasma, or if it can be released into the extracellular environment upon cell activation. A general feature in cell signaling processes is that a signal, such as ceramide or C1P, is generated by the action of an enzyme or a group of enzymes acting in concert. The enzyme product will then activate specific downstream electors leading to a cascade of metabolic reactions that will end up stimulating or inhibiting a biological function. Therefore, further studies need to clarify these points. Although practical applications could not be reached at the moment, the development of inhibitors or activators of the enzymes that affect the intracellular

concentrations of C1P may be crucial for establishing therapeutic strategies; furthermore, administration of exogenous C1P, synthetic analogues of C1P, as well as C1P receptor agonists are other potential approaches.

4.2. Peroxiredoxin I

In chapter 3, we revealed that Prx I may play a protective role of cochlear lateral wall against cisplatin toxicity. This finding highlights a potential usefulness of Prx I as a protective factor that could ameliorate cisplatin-induced side effects on hearing function. The up-regulation of Prx I in cells and tissues under oxidative stress conditions is either enhancing the cellular defense capacity against oxidative damage or facilitating the recovery process from oxidative damage. Therefore, understanding mechanism of this up-regulation might have to shed a light on proper strategies to deal with oxidative stress of cisplatin. However, it is also worth to note that up-regulation of Prx I after oxidative damage, is likely to act in concert with other Prxs and the realm of other antioxidant enzymes and proteins, thus contributing in more or less specific ways to overall response to oxidative challenges.

The contribution of oxidative stress in human disease states has long been recognized, and bolstering of antioxidative pathways may benefit to prevent chronic diseases, such as cardiovascular diseases, cancer, diabetes mellitus, and so on. However, we do have a lot of questions remaining unanswered in this field. For example, what directly or indirectly affects Prx synthesis, release, and breakdown is still not well understood. What pharmacologic or non-pharmacologic treatment interventions can modulate Prx levels are also unclear currently. However, what we could do is to find the association between their levels and severity of a related disease as the first step in this long-term plan; therefore,

we could determine whether it is a possible biomarker for diagnosis or prognosis a disease.

4.3. Concluding remarks

Although many studies concentrate on cisplatin ototoxicity, there are a lot of unanswered questions in this filed, and the best way to find a suitable toxic treatment is to fully understand its mechanism. In our study, we suggested that C1P, ceramide kinase or Prx I could be a possible target in dealing with cisplatin ototoxicity. An effective method to reduce cisplatin ototoxicity is still waiting ahead, and we hope our findings will build a small step to archive it soon.

Chapter 5. Acknowledgements

Firstly, I would like to express my appreciation to professor Osamu Ohneda who gave me the opportunity to pursue my Doctoral degree in the University of Tsukuba. His substantial supports helped me so much during my Ph.D study.

Besides, I sincerely express my gratefulness to professor Akira Hara who accepted me to work in the Otolaryngology laboratory. I always appreciate his kindness, his valuable advice and his wonderful supports. Also, I would like to thank Dr. Keiji Tabuchi for important contributions to my research. Without his precious supports, it would not be possible to conduct this research.

In addition, I would like to thank the thesis committee: professor Yoshito Kumagai, professor Tetsuro Oshika, professor Masayuki Matsumoto, and assistant professor Tomoko Yamada for their insightful comments and important questions which help me to improve my thesis from various perspectives.

I would like to thank members of the Department of Otolaryngology and my lab mates for always treating me very nice and giving me the best encouragements. I always keep in my minds the wonderful times we had together.

My sincere thanks also goes to members of the International office, Medical department office, Academic Service Office for the medical Science Area, and International Student Center of University of Tsukuba for providing information and kind supports.

I would like to thank Japan Society for the Promotion of Science and Mitsubishi Foundation Scholarship for providing financial support during my Ph.D study.

I also thank to all of my international friends and Vietnamese friends. They are always readily there to help me, to encourage me and to lift me up to overcome tough time or to share happy moments. They are a part of my life now and forever.

From the bottom of my heart, I dedicate my work to my father Le Phuong, and my mother Le Thi Khang for their unconditional and endless love. They are my pride, my strength and my power forever. It also goes to my brother Le Vinh who has taken care of my mother while I was studying in Japan, and to my other family members who always give a lot of supports.

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