Original Article

Modulation of copper transporters in protection against cisplatin–induced cochlear hair cell damage

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Abstract Cisplatin belongs to platinum–based drugs and is widely used in cancer chemotherapy. Ototoxicity is one of the major dose limiting side--effects of cisplatin. For toxicity to occur cisplatin must first be transported from the bloodstream into cochlear cells. Three copper transporters are considered pathways for regulating the uptake and translocation of cisplatin into cells: Ctr1, ATP7A and ATP7B. Our recent study with cochlear organotypic cultures shows that cochlear hair cells can be destroyed by cisplatin at low concentrations from10μm to 100μm. However, high doses of cisplatin cannot damage hair cells, maybe due to intrinsic feedback reactions that increase export of platinum by ATP7B when the platinum concentration is high in extracellular space. Cimitidine is a specific copper transporter inhibitor that can block the entrance of copper and platinum, and may prevent cisplatin–induced cochlear hair cell injury. To evaluate this hypothesis, we treated cochlear organotypic cultures with cisplatin (10 μm or 50 μm) alone, or cisplatin combined with cimitidine at concentrations ranging from10–2000 μm for 48 hours. cisplatin at 10 μm damaged about 20% hair cells. In contrast, when cimitidine (10 μm, 100 μm and 2000 μm) was added to the culture, near 100% cochlear hair cell survived. At higher concentration (50 μm), cisplatin destroyed about 80% of cochlear hair cells. However, 100 μm cimitidine rescued about 50% hair cells from cisplatin damage, and 2000μm cimitidine protected about 80% hair cells. The data of western blot showed that CTR1 and ATP7B expressions were increased in cisplatin treated cochlear tissue, but cimitidine significantly reduced CTR1 and ATP7B. In addition, ATP7A expression was depressed a little after cisplatin treatment. Considering that Ctr1 is involved in copper and platinum influx, but the ATP7A and ATP7B are copper export transporters, the results suggest that cimitidine can effectively block the entrance by copper transporters and stop the influx of cisplatin.

Keywords cisplatin, copper transporter, ototoxicity, cochlear hair cell

Introduction

Cisplatin is one of the most widely used chemotherapeutic agents for the treatment of various malignant tumors. The antitumor effects of cisplatin largely arise from intrastrand and interstrand cross linking of DNA leading to G2 cell cycle arrest thereby blocking tumor proliferation.1, 2 Cisplatin also induces a complete blockage at the S phase of the cell cycle inhibiting total mRNA transcription.3

In addition to its potent anti–tumor actions, cisplatin is highly toxic to the kidney, liver, nervous system, bone marrow, inner ear, etc.4–10 The ototoxic effects of cisplatin produce significant hearing loss and permanent destruction of cochlear hair cells and spiral ganglion
neurons (SGNs). Cisplatin–induced damage to cochlear hair cells (HCs) is initiated in outer hair cells (OHC) followed by inner hair cells (IHC). The HC damage begins at the basal turn of the cochlea and gradually spreads to the apex in vivo. However, cisplatin destroys both OHCs and IHCs evenly through the entire length of the cochlea in vitro. Besides the damage to HCs, cisplatin also damages SGNs in the modiolus, and the marginal epithelium on the stria vascularis. Therefore, HCs, SGNs and stria vascularis are three major targets of cisplatin toxicity in the cochlea.

The ototoxic mechanisms of cisplatin involve multiple factors, such as generation of highly toxic free radicals and reactive oxygen species (ROS) with reduction of antioxidant enzymes, destruction of DNA, damage of mitochondria, activation of caspase cascade, and activation of p53. Although a complicated network of apoptotic signals is involved in cisplatin ototoxicity, the major apoptotic pathway induced by cisplatin in the cochlea is initiated from cell death receptors on the cell membrane, and then converged to p53 signaling pathway to complete the programmed self–destruction finally. For toxicity to occur cisplatin must first be transported from the bloodstream into cells. Cisplatin can be activated once it enters the cytoplasm when the chloride atoms on cisplatin are replaced by water molecules. The aquatic cisplatin becomes potent electrophile and then react with nucleic acid to target DNA. In addition, previous studies have demonstrated that when cisplatin is transported into the cell, it can bind with glutathione, and then becomes a cisplatin–glutathione complex to exert its toxic effects. Therefore, the entry of cisplatin is the first important step responsible for its intracellular toxic effects.

The pathways that regulate the uptake and translocation of cisplatin into HCs, SGNs, and cochlear supporting cells are poorly understood. However, research evidence suggests that cisplatin uptake is mediated by copper transporters that mediate copper and platinum–based compounds from cytoplasm. Cells over expressing ATP7B show increased efflux of cisplatin and carboplatin from the cytoplasm and reduced accumulation of these compounds. These results suggest that ATP7B is important in drug efflux and cisplatin resistance. In contrast, cells that over express ATP7A accumulate large amounts of cisplatin in vesicles. These cells are also resistant to toxicity presumably by preventing escape of platinum to potentially lethal targets such DNA. Therefore, Ctrl, ATP7A, and ATP7B regulate the uptake and extrusion of copper and platinum–based compounds.

The efficiency of copper and/or platinum input or output depends on the requirement of the cell and the level of intracellular copper/platinum. Cells may detect intracellular and extracellular copper/platinum levels for the appropriate regulation. If the level of copper in the cells is high, cell can enhance its export; otherwise it may gain its import. For example, high extracellular copper leads to the endocytosis of Ctrl from the membrane and subsequent protein degradation. This reduces the uptake of copper/platinum from the extracellular environment preventing excess copper uptake into the cytoplasm. These important findings suggest that high level of extracellular copper/platinum is detectable by the cell, which causes Ctrl to rapidly withdraw from the membrane into the cytoplasm where Ctrl is quickly degraded; and that this negative feedback mechanism reduces the uptake of copper/platinum and reduces its toxicity.

In order to test if HCs can also regulate input and output of cisplatin to achieve a self–defense, post–natal cochlear organotypic cultures were treated with various doses of cisplatin for 24 or 48 hours. The missing of HCs and the changes in expression of copper transporters by cisplatin treatment was evaluated in present study. In addition, the protective effects of copper transporter inhibitor, cimetidine was also evaluated to illuminate the possibility of regulating copper transporters.

Materials and Methods
Cochlear organotypic cultures and cisplatin treatment
Rat pups (Sprague Dawley) at postnatal day 3 were
used for this project. The cochlea was carefully removed in Hank’s Balanced Salt Solution (1X GIBCO, 14175, Psisley, U.K.). The lateral wall of the cochlea was dissected away and the whole basilar membrane containing the organ of Corti and spiral ganglion neurons was taken as a flat surface preparation under a microscope. A drop (12 μl) of rat tail collagen (Type1, BD Biosciences, 4236 Bedford, MA), 10×basal medium eagle (BME, Sigma B9638), 2% sodium carbonate, 9:1: 1 ratio) was placed in the center of a 35 mm diameter culture dish (Falcon 1008, BectonDickinson) and allowed to gel at room temperature. After the gel became gelled, 1.2 ml of serum–free medium consisting of 2 g bovine serum albumin (BSA, Sigma A–4919), 2 mlSerum–Free Supplement (SigmaI–1884), 4.8 ml of 20% glucose (Sigma G–2020), 0.4 ml penicillin G (Sigma P–3414), 2 ml of 200 mM glutamine (Sigma G–6392), and 190.8 ml of 1X BME (Sigma B–1522) were added into the dish. The cochlear tissues were placed on the surface of the collagen gel. The cochlear explants were placed in an incubator (Forma Scientific, #3029) and maintained at 37°C in 5% CO2 overnight. On the second day, the cochlear tissue was treated with various experimental conditions for 24 or 48 hours.

Experimental conditions

After overnight recovery, cochlear explants were treated with various concentrations of cisplatin (0, 10, 50, 100, 400, and 1000 μm) for 24 or 48 h for cisplatin dose response evaluation.

To test if copper transporter blocker, cimetidine can prevent entry of copper transporter to protect cochlear hair cells against cisplatin injury, cochlear explants were treated with different concentrations of cimetidine (10, 100, 2000, and 4000 μm) for 24 or 48 h with or without cisplatin (10, 50 μm).

Histology

In the end of the experiment, the cochlear tissue was fixed with 10% formalin in PBS for 4 hours. After fixation, specimens were rinsed in 0.1M PBS, immersed in TRITC–labeled phalloidin (Sigma P1951, 1:200) in PBS for 30 min. Specimens were rinsed three times in 0.1M PBS and mounted on glass slides in glycerin. Under a fluorescent microscope (BioRad MRC1024), specimens were examined with appropriate filter for TRITC (absorption: 544 nm, emission: 572 nm). The number of missing OHC and IHC for each 0.24–mm segment was counted through entire the length of the organ of Corti. A cochleogram was constructed by plotting the percent IHC loss and OHC loss as a function of percent distance from the apex of the cochlea. To aid in the analysis, average cochleogram was constructed from specimens for each experimental condition. Because each end of the basilar membrane was damaged during surface preparation, comparisons between HC counts were made between 20% and 80% of the distance from the apex of the cochlea.

Western Blotting

Western blots were used to assess the level of protein expression of Ctr1, ATP7A and ATP7B from cochlear explants exposed to the various experimental conditions described above (i.e., dose–damage response experiments: control, 10, 50, 400 and 1000 μm cisplatin; otoprotective experiments: control, cisplatin (10 and 50 μm), cimetidine (2000 μm, and cisplatin + cimetidine). To provide sufficient protein for analysis, 20 cochlear explants were used for each treatment condition. Protein concentrations were measured by the Bradford assay (Bradford, 1976). Proteins from crude lysates (40 μg protein) were separated by SDS–polyacrylamide gel electrophoresis using precast 12.5% NuPAGE gels (Invitrogen) at 200V according to the manufacturer’s guidelines. Proteins were transferred to polyvinylidene difluoride membranes, blocked with 0.2% 1–Block (Applied Biosystems, Foster City, CA), 0.1% polyethylene glycol sorbitan monolaurate (Twee 20, Sigma) in PBS, and probed by incubation for 1 h with primary antibodies for Ctr1, ATP7B, or ATP7A. Membranes were washed in PBS–Twee (0.1% Twee 20) and incubated for 1 h with an appropriate HRP–conjugated secondary antibody (Pierce Chemical Co., Rockford, IL) in blocking buffer. After washing in PBS–Twee, antibody complexes were visualized by chemiluminescence using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce) and a Fuji LAS–1000 Plus chemiluminescence imager (Tokyo). Figure panels were made by digitally assembling the relevant lanes with Adobe Photoshop software. Background corrected bands (NIH Image J software) were normalized against bands obtained by stripping the membrane with 25 mM glycine (BioRad, Hercules, CA), pH 2.0, 1% lauryl sulfate (Fisher Scientific,
Pittsburgh, PA) and reprobing with an antibody against glyceraldehyde 3 phosphate dehydrogenase (GAPDH, Millipore). GAPDH was used as a loading control as the housekeeping protein to compare with specific bands of the Ctrl1, ATP7B or ATP7A. To quantify the changes in Ctrl1, ATP7B or ATP7A expression, we calculated the ratio of the relative density of Ctrl1, ATP7B or ATP7A bands compared to the GAPDH. The relative density ratio of Ctrl1:GAPDH, ATP7B: GAPH, ATP7A: GAPH in the control condition was set at 1.0, and the ratios of densities corresponding to the other treatment conditions were normalized accordingly.

Results

**HC degeneration after cisplatin treatment**

After 24 hours of culturing with various doses of cisplatin, there was no HC loss. However, the stereocilia bundle showed disarray or missing in low dose cisplatin treated cochleae (10 μM, 50 μM and 100 μM). In contrast, the stereocilia bundle presented normal when the concentration of cisplatin exceeded 400 μM (Figure 1).

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**Fig. 1** After 24 h of cisplatin treatment with various doses showing no HC loss. However, disarrayed stereocilia bundles are seen in low dose cisplatin treated cochleae (10 μM, 50 μM, and 100 μM), while the stereocilia bundle presents normal in high concentration cisplatin treated cochlear explants (400 μM and 1000 μM). Arrowheads point to pictures of HCs at the corresponding locations on the cochlear basilar membrane near the apical turn.
After 48 hours of culturing, hair cell damage appeared in lower dose cisplatin treated cochlea (10 μm, 50 μm, and 100 μm). However, most HCs were intact with the high doses of cisplatin treatment (400 μm and 1000 μm, Figure 2). The cochleograms revealed an unusual dose–damage response relationship: hair cell loss increased up to 50 μm cisplatin, but then dropped off precipitously at doses higher than 100 μm.

**Changes in expression of copper transporters following cisplatin treatment**

Western blots were used to quantify the changes in the level of protein expression of Ctr1, ATP7B, and ATP7A following exposure to cisplatin at various concentrations (0, 10, 50, 400 and 1000 μM cisplatin). As shown in Fig. 3, the expression of Ctr1 was relatively unchanged by cisplatin doses that induced appreciable hair cell loss (i.e., 10 and 50 μM), whereas there was a modest (~3-fold) increase in the expression of Ctr1 at the high cisplatin doses (400 μM and 1000 μM). In contrast, a dramatic cisplatin dose–dependent increase was detected in the expression of ATP7B. Compared to the control condition, there was an over 120–fold increase in the expression of ATP7B following 1000 μM cisplatin exposure. The expression of ATP7A was increased visibly at dose of 50 μM that induced hair cell loss, while a great reduction was seen when the concentration of cisplatin exceeded 400 μM (Fig. 3).
To determine the safest endurance limit of cimetidine to the HCs, the specimens were observed along the entire length of the organ of Corti for different doses of cimetidine treatment. Figure 4 shows the morphological changes in pattern of stereocilia of HCs 48 h after cimetidine treatment at various doses ranging from 10 to 4000 μm. The pattern of stereocilia bundle on HCs presented normal in 10 μm, 100 μm, and 2000 μm cimetidine treated cochleae (Fig. 4A, 4B, and 4C). However, when the concentration of cimetidine reached 4000 μm, the stereocilia of hair cells were disorganized (Fig. 4D), despite the fact that there was no hair cell missing, suggesting that 48 h treatment of 4000 μm cimetidine under culture condition may cause potential injury to HCs, whereas 2000 μm cimetidine treatment may be safe to HCs.

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**Fig. 4** Microphotographs of representative cultures labeled with TRITC–conjugated phalloidin. (A) cochlear explants treated with 10 μm cimetidine for 48 h. (B) cochlear tissue treated with 100 μm cimetidine for 48 h. (C) cochlear explants treated with 2000 μm cimetidine for 48 h. (D) cochlea treated with 4000 μm cimetidine for 48 h. Note that treatment with 4000 μm cimetidine results in disarrayed stereocilia bundle.
Protective effects of cimetidine to HCs against cisplatin

Figure 5 and figure 6 show the protective effect of cimetidine in preventing cisplatin injury to HC. The addition of 2000 μm cimetidine to the cochlear cultures enhanced HC survival. Treatment of cultures with 10 μm cisplatin alone damaged about 20% of the HCs (Fig. 5A, 6A). When the cultures were treated with cisplatin plus cimetidine (10, 100, or 2000 μm), nearly 100% of HCs survived (Fig. 5B, 5C, and 5D, Fig. 6A). Treatment of cochlear cultures with 50 μm cisplatin resulted in approximately 90% HCs loss (Fig. 5E, Fig. 6B) and addition of 10 μm cimetidine did not protect HC (Fig. 5F, Fig. 6B). However, when the cultures were treated with cisplatin plus 100 μm of cimetidine, HC loss decreased to about 50% (Fig. 6B). When the concentration of cimetidine was increased to 2000 μm, HC loss decreased to 20% (Fig. 5G, 5H, Fig. 6B).

Fig. 5 Microphotographs of phalloidin-labeled cochlear organotypic cultures. (A) cochlear explants treated with 10 μm cisplatin for 48 h with mild HC loss. (B) Intact HCs when the cochlear culture is treated with 10 μm cisplatin plus 10 μm cimetidine for 48 h. (C) No HC missing when cochlear explants is treated with 10 μm cisplatin plus 100 μm cimetidine for 48 h. (D) HCs present normal when treated with 10 μm cisplatin plus 2000 μm cimetidine for 48 h. (E) cochlear explants treated with 50 μm cisplatin plus 10 μm cimetidine for 48 h with severe HC loss. (F) HCs cannot be rescued when the cochlear tissue is treated with 50 μm cisplatin plus 100 μm cimetidine for 48 h. (G) HC loss is decreased to about 50% when treated with 50 μm cisplatin plus 100 μm cimetidine for 48 h. (H) cochlear explants treated with 50 μm cisplatin plus 2000 μm cimetidine showing HC survival of about 80% (Fig. 5G, 5H).

Fig. 6 Protective effects of cimetidine to HCs when co-administered with cisplatin. The density of surviving HC is measured between 20% and 80% of the distance from the apex of the cochlear explants (n=6 per condition) that have been exposed for 48 h to 10 μm or 50 μm cisplatin with or without cimetidine (10, 100 or 2000 μm). HC density is reduced by about 20% when treated with 10 μm cisplatin, but most HCs are intact when cimetidine (10, 100, or 2000 μm) is added (A). More than 80% HCs are destroyed by 50 μm cimetidine, but increasing the dose of cimetidine to 100 μm results in an increase of survival HC to 50% , while adding 2000 μm cimetidine rescues about 80% of HCs. Cimetidine at 10 μm does not protect the HC from cisplatin damage (B).

Changes in expression of copper transporters following cisplatin/cimetidine treatment

Western blots showed that cisplatin treatment alone increased the expression of Ctr1 and ATP7B, while cimetidine administered alone reduced the expression in both Ctr1 and ATP7B. However, the expression of ATP7A showed little changed (Fig. 7). When the cochlear explants were treated with cisplatin plus cimetidine, the expression of Ctr1 and ATP7B was still lower than normal (Fig. 7).

Discussion

The ototoxic effects of cisplatin in vivo study often result in HC lesions with OHC damage being more severe than IHC, and the HC loss progresses along a base to apex gradient, which is very similar to most ototoxic reagents, such as aminoglycoside antibiotics. However, an unusual hair cell lesion was found in cisplatin treated cochlear cultures in that the IHC and OHC destruction is nearly the same along the entire length of the cochlea. This may suggest that when the cochlear tissue is displaced into culture conditions, the cellular response to cisplatin ototoxicity is different from in vivo conditions.
Copper transporters are the major cellular entrance of copper and platinum. Information from cancer research has revealed that cells can adjust the copper/platinum import and export natively by action of copper transporters. In vitro studies on nephrotoxicity of cisplatin have confirmed that the concentrations of cisplatin up to 1.67 mM maintain a dose-dependent manner. In our recent study, a dose-dependent response was seen in RB142 cell lines in various cisplatin concentrations up to 1000 μm. The ototoxic effect of cisplatin in Zebrafish is also dose-dependent at high concentrations up to 400 μm. These may indicate that the chemical property of cisplatin in cellular damage does not alter at high concentrations. Interestingly, an incidental finding was disclosed in current study that when the concentration of cisplatin exceeds 400 μm, HCs show an intrinsic resistance to cisplatin. Although previous reports alleged that cisplatin–induced ototoxicity showed a dose-dependent manner, the highest cisplatin concentration applied in mammalian cochlear cultures was under 10 μg/ml (33.3 μM). Therefore, previous dose dependent response of cisplatin was restricted to the low level of cisplatin. In the current study, the greatest destruction of HCs was found at low concentrations ranging from 10 μm to 100 μm. In contrast, high dose of cisplatin ranging from 400 μm to 1000 μm did not demolish the HCs. The changes in expression of copper transporters revealed that high dose cisplatin resulted in a significant increase of copper/platinum efflux by ATP7B, which effectively decreased the intracellular platinum accumulation. Thus, we ascribe the limited ototoxicity observed at high doses of cisplatin to a reduction of intracellular accumulation via increased ATP7B–mediated efflux. The dynamic changes in expression of ATP7A may reflect the changes in platinum accumulation by great deal of platinum efflux caused by ATP7B. However, if the dose of cisplatin is low, HC may relax vigilance allowing platinum to enter the cell.

As a potent copper transporter inhibitor, cimetidine can effectively prevent the action of copper transporters without interfering with the antitumor effects of cisplatin. In our recent study, we have demonstrated that cimetidine has a potential to protect HC against cisplatin toxicity. The similar protective effects have been repeated in the current study. The result from western blot in present study further demonstrated that cimetidine can play an important role in HC protection. Considering the exporters of copper transporters, ATP7A and ATP7B were also inhibited by cimetidine. The protective effects of cimetidine to HC should be credited to its inhibiting the influx of cisplatin through copper transporter importer, Ctr1.

Cisplatin ototoxicity seriously affects people’s quality of life. To prevent the ototoxicity of cisplatin, many strategies can be considered for inner ear protection. For example, antioxidation and anti-apoptosis are partially efficient in protecting HCs against cisplatin ototoxicity. The premise for those protections must be in the process of cell degeneration. Therefore, the protective actions in both antioxidation and anti-apoptosis are limited. The results in the current study by modulating copper transporters may lead to development of a new strategy against cisplatin ototoxicity, which keeps cisplatin out of the cell by reducing platinum influx and enhancing platinum efflux.
References