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Use of Organotypic Cultures of Corti's Organ to Study the Protective Effects of Antioxidant Molecules on Cisplatin-Induced Damage of Auditory Hair Cells

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Hypothesis: Cisplatin causes the generation of reactive oxygen species (ROS), which interferes with the antioxidant defense system of Corti's organ and results in damage to the hair cells.
Background: Cisplatin is a widely used chemotherapeutic agent with the dose-limiting side effect of ototoxicity. Evidence is accumulating that cisplatin interferes with the antioxidant defense system of Corti's organ.
Methods: Organotypic explants of P-3 rat organ of Corti were the in vitro model system. Presence of intact auditory hair cells and stereocilia bundle integrity was assayed by phalloidin-FITC staining. Fluorescent dye probes detected H_2O_2 and intracellular thiol [e.g., glutathione (GSH)]. Spectrophotometric analysis determined antioxidant enzyme levels.
Results: There was a rapid dose-dependent cisplatin cytotoxicity in the explants after 48 h of exposure. An accumulation of H_2O_2 and a reduction of GSH levels were observed within cisplatin-exposed hair cells. L-buthionine sulfoximine, an inhibitor of GSH formation, enhanced cisplatin ototoxicity, whereas N^6-(2-phenylisopropyl) adenosine, an adenosine agonist, elevated antioxidant enzyme levels and ameliorated cisplatin toxicity. The following molecules protected hair cells from cisplatin-induced damage: GSH; glutathione diethyl ester (GSHe); ebselen (EBS); 4-methylthiobenzoic acid (MTBA); and D-methionine (D-MET). EBS, MTBA, and D-MET in vitro protection correlates with in vivo protection in rats.
Conclusions: Organotypic culture of Corti's organ has been validated as a model for studying cisplatin toxicity and for screening otoprotective molecules. Some of the events that contribute to cisplatin's ability to damage auditory hair cells are generation of ROS (e.g., H_2O_2), depletion of intracellular GSH, and interference with antioxidant enzymes within the cochlea. Agents that bolster the cochlea's antioxidant system can prevent cisplatin destruction of auditory hair cells. Identified protective agents may prove to be clinically useful in limiting or completely protecting from cisplatin ototoxicity. **Key Words:** Antioxidant molecules—Auditory hair cells—Cisplatin—Corti's organ—Organotypic cultures.
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Cisplatin is a widely used chemotherapeutic agent for treating both adult (1–4) and pediatric (5–7) malignancies. One of the dose-limiting side effects of cisplatin is the occurrence of a bilateral, dose-dependent, progressive, irreversible sensorineural hearing loss, which begins in the high frequencies but can progress to lower (speech) range frequencies (1–7). The incidence of hearing loss varies substantially with the cumulative dose of cisplatin, method of administration, age of the patient, exposure to either cranial irradiation or other ototoxins, and existence of previous cochlear damage (1–7). Because clinical studies have used a wide variety of criteria to establish and measure the amount of hearing loss caused by cisplatin exposure, the reported incidence of hearing loss varies widely. A range of hearing loss of 20–90% has been reported for adults (1–4) and a range of 50–90% loss has been reported for children receiving cisplatin therapy (5–7). An inability to understand speech

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and tinnitus are frequent clinical complaints (2). As renal toxicities of cisplatin are reduced, ototoxicity becomes a primary dose-limiting factor (4).

The range of morphologic effects in the cochleae of cisplatin-exposed animals (8–12) and humans (13) have been described and include damage to the outer and inner hair cells (IHCs), auditory neurons, stria vascularis, and organ of Corti supporting cells. The pattern of hair cell damage has been shown to progress from base to apex and from the third row of outer hair cells (OHCs) to the first row of OHCs and then to the IHCs (8,10–13). With low levels of cisplatin exposure, first hair cell stereocilia tip-links are damaged, followed by disorganization and finally fusion of stereocilia (14). As cisplatin dosages increase, both mitochondria and the endoplasmic reticulum are damaged, followed by a loss of stereocilia and then loss of hair cells (8–13). With even higher doses of cisplatin, there is atrophy of the stria vascularis, collapse of Reissner's membrane, and damage to the support cells of Corti's organ (8,11).

Although the morphologic changes in response to cisplatin toxicity have been defined, the molecular mechanisms of cisplatin auditory hair cell damage are not completely understood. Theories of toxicity include inhibition of a key enzymes such as adenosine triphosphatase, adenylate cyclase, cytochrome P-450 microsomal enzymes, glucose-6 phosphate dehydrogenase; alteration in lysosomal enzyme activities; disruption of mitochondrial oxygen transport; and/or disruption of calcium homeostasis (13).

Evidence is currently accumulating that cisplatin exerts its renal and cochlear toxicity by generating reactive oxygen species (ROS) and interfering with the antioxidant defense system of these organs (15–21). Antioxidant enzymes as well as smaller molecules that scavenge and modify ROS act to limit cellular damage (22). This system protects eukaryotic cells not only from ROS such as H_2O_2 but also from damage caused by free radicals, i.e., compounds with unpaired electrons. Such compounds include hydroxyl, superoxide, lipid peroxide, divalent metals, and others. ROS are generated through normal cellular metabolism, ischemia-reperfusion injury, inflammation, irradiation, and chemotherapeutic agents, as well as by some antibiotics (22). If left unchecked, ROS once generated can react with a variety of cellular components such as protein, DNA, and unsaturated lipids, leading to chemical modification and to metabolic and structural alterations, which can lead to cell death (22).

The antioxidant defense system consists of cytosolic and membrane-associated small molecules and a variety of enzymes involved in oxidation-reduction pathways and the gamma-glutamyl cycle, which scavenge or modify hydrogen peroxide and free radicals to prevent cell damage. The small molecules of the antioxidant defense system include vitamin E, beta carotene, ascorbate, and glutathione (GSH) (22). GSH is an essential thiol-containing tripeptide found in nearly all animal cells and acts as a free radical scavenger, functions as a reducing agent to keep key enzymes in an active state, and keeps other free radical scavengers such as ascorbate in an active (reduced) state (23). A number of important antioxidant enzymes that have been found to be active in cochlear tissues include superoxide dismutase, catalase, GSH reductase, GSH peroxidase, glutathione-S-transferase, and glutamyl cysteine synthetase (a rate-limiting enzyme for GSH synthesis) (20,21,24–26). Through the action of reductases, peroxidases, and glutathione-S-transferases GSH participates in the metabolism and detoxification of electrophilic drugs, metabolites, and ROS (20–23).

Rybak et al. have provided indirect evidence that ROS are involved in cisplatin ototoxicity (20,21). In acute in vivo experiments where rats were exposed to high doses of cisplatin, these investigators noted a significant decrease in the activity of GSH reductase and peroxidase, as well as in GSH, and an increase in superoxide dismutase and catalase activity in cochlear tissues. Additionally, these investigators noted the accumulation of malaondialdehyde, a marker for cell membrane lipid oxidative damage (20,21).

We used organotypic cultures of Corti's organ from 3-day postpartum (P-3) rats to further study the effects of cisplatin on auditory hair cells. Real-time confocal microscopic analysis of specific fluorescent dye probes allowed the study of the formation of H_2O_2 and the modulation of intracellular GSH levels of auditory hair cells in live explants. Spectrophotometric analysis of isolated cochlear duct extracts determined antioxidant enzyme activities in the explants. The antioxidant defense system of the explants was either inhibited by addition of L-buthionine sulfoximine (BSO), an inhibitor of GSH synthesis, or augmented by exposure to N^6-(2-phenylisopropyl) adenosine (RPIA), an inducer of antioxidant enzyme activity. Finally, several antioxidant molecules were screened for their effectiveness in preventing cisplatin-induced hair cell destruction and stereocilia bundle damage in vitro.

METHODS

Organotypic cultures

Three-day postpartum rats (Wistar, Charles River, Wilmington, MA) were killed and decapitated in accordance with National Institutes of Health guidelines. Cochleae were prepared in sterile Dulbecco's phosphate-buffered saline (PBS, pH 7.2). Organ of Corti explants were excised from the modiolar tissue, placed in individual wells of a 96-well plate in 100 µl of Dulbecco's modified Eagles medium with high glucose (6 g/L) and an N1 cocktail of supplements (27) and incubated at 37°C in 5% CO_2 and room air.

Hair cell quantification and stereocilia analysis

Specimens for morphologic analyses were fixed for 1 h in freshly prepared 4% paraformaldehyde at room temperature. Cochlear ducts were then stained with florescein isothiocyanate (FITC)-conjugated phalloidin (FITC-phalloidin; Sigma, St. Louis, MO) as previously described (27). FITC-phalloidin–stained explants were observed using a Zeiss Axiophot epifluorescent microscope with a blue excitation filter set (450–490 nm) objective lens. Segments of cochlear duct were sampled

from each of the cochlear turns with IHCs and OHCs quantified per 0.1 mm of cochlear duct length using an ocular grid system. Using a 20× objective lens, FITC-phalloidin–stained intact cuticular plates were counted to determine hair cell density. Stereocilia morphology was analyzed using a 40× objective lens with the integrity of stereocilia bundles from 10 IHCs and 30 OHCs scored for each cochlear turn. Stereocilia bundle integrity was characterized as either normal, disorganized, or fused/absent with a stereocilia bundle integrity index calculated as a mean of intact (normal or disorganized) stereocilia bundles.

Dose response curves

Three to four organ of Corti explants were exposed to cisplatin (Platinol, Bristol Meyers Squibb, Princeton, NJ) at a concentration of either 4, 6, 8, 10, or 12 μg/ml. Other groups of explants (n = 3 to 4 per group) were exposed to the same concentrations of cisplatin with the addition of either BSO (Sigma), RPIA (Sigma), L-N-acetyl-cysteine (L-NAC; Sigma), or L-NAC plus RPIA. Concentrations used were as follows: BSO, 5 μmol/L; RPIA, 100 μmol/L; L-NAC, 10 mmol/L. BSO, L-NAC, or RPIA were added to the cultures during the initial 24 h in vitro. Cisplatin, either alone or in combination with other molecules (e.g., BSO, RPIA, L-NAC), was added to the explants after an initial 24 h of culture. Exposure to cisplatin or cisplatin with other compounds (e.g., cisplatin—L-NAC) was for 48 h, resulting in a total of 72 h in vitro. After 72 h of culture, the explants were fixed and stained with FITC-phalloidin as described, and hair cells and/or stereocilia morphology was quantified (Fig. 1). There were three to four explants for each concentration and treatment group, and each experiment was repeated three times with similar results.

Protectant molecules

The following molecules were tested for their effectiveness in reducing cisplatin-induced hair cell damage in vitro: reduced GSH (Sigma), glutathione diethyl ester (GSHe, synthesized by Drs. Blanchard and Binder), L-NAC (Sigma), 4-methylthiobenzoic acid (MTBA; Aldrich Chemicals, Milwaukee, WI), D-methionine (D-MET; Sigma), and ebselen (EBS; ICN Pharmaceuticals, Inc., Costa Mesa, CA, U.S.A.). The GSHe was prepared as outlined by Levy et al. (28). Purity was assessed by derivatizing the sample with 5, 5^1 dithiobis-ra-nitro benzoic acid (DTNB) (Ellmen's reagent) and injecting the material into a C_{18} reverse-phase high-performance liquid chromatography column. Elution with a methanol gradient and detection at 325 nm allowed the purity of the sample to be estimated as >90%, with the remainder being monoethylester of GSH (8–10%) and a trace of unmodified GSH. The experimental scheme for the evaluation of protectant molecules is presented in Fig. 1 (n = 7 to 9 explants per treatment group).

Real-time confocal analyses of GSH and ROS levels in organ of Corti explants

Control, cisplatin-exposed, or cisplatin-exposed protected (e.g., GSHe, 10^{-2} mol/L) cochlear duct explants were double-labeled with either membrane-permeant monochlorobimane (MCB, 40 μmol/L, Molecular Probes) or membrane-permeant 2′-7′-dichlorodihydrofluorescin (DCF) applied as a diacetate (2 μmol/L, Molecular Probes) plus calcein blue AM (40 μmol/L, Molecular Probes) for 20 min at 37°C. DCF is nonfluorescent but upon exposure to H_2O_2 is converted by cellular enzymes to the brightly fluorescent dichlorofluorescein (29,30). MCB reacts with reduced intracellular thiols, the vast majority of which are GSH molecules, to produce a fluorescent reaction product with the level of fluorescence being proportional to the intracellular GSH content (30). Calcein blue AM fluorescence preferentially stains hair cells and therefore, was used as a marker for auditory hair cells. Cochleae were examined at 2, 4, 8, 12, 24, or 36 h after exposure to cisplatin, or cisplatin plus protectant, with a Nikon real-time RCM8000 scanning confocal microscope equipped with an Argon laser optimized for the 351 nm line, using appropriate excitation wavelengths for the various probes, and a 40× water immersion, ultraviolet light–corrected objective lens. The following excitation wavelengths were used: calcein blue AM and MCB, 351 nm; DCF, 488 nm. The emitted light after excitation for all of the dye probes was 515 nm. Neutral density filter settings and pinhole size were held constant for DCF and MCB imaging, respectively, for all samples at all time points. Images were stored on optical disk for later analysis. Four separate fields in each of 60–65 explants were examined in each confocal experiment, which was repeated three times with similar results.

Enzyme activity analysis

All explants for enzyme studies were cultured for an initial 24 h in control medium and then cultured for the next 24-h period in either control medium; cisplatin, 10 μg/ml; or RPIA, 100 μmol/L. A second set of explants received an initial 24-h culture period in control medium, followed by a 48-h period of culture in either control medium or cisplatin, 10 μg/ml. After either 48 or 72 h, in vitro organ of Corti explants were rinsed in PBS, and then frozen at −70°C until extract preparation. Frozen cochlear explants were thawed then sonicated and centrifuged at 10,000 rpm for 20 min at 0°C, and the supernatant was collected for analysis (25). Supernates were analyzed for the following enzyme activities: catalase, glutathione reductase (GSHred), gamma glutamyl cysteine synthetase (γGCS), and glutathione-S-transferase (GSH S-T). Catalase was assayed by directly monitoring the decomposition of H_2O_2 (31) with specific activity expressed as μmol/L H_2O_2 decomposed/min/mg

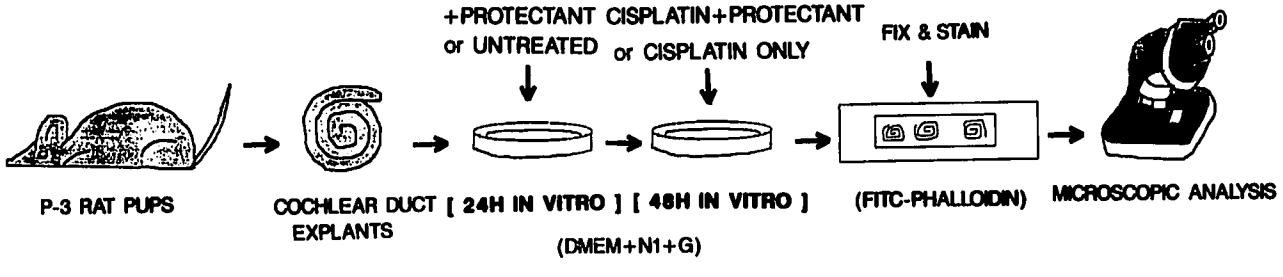

FIG. 1. Schematic representation of the in vitro protocol for analyzing otoprotective molecules.

of protein (determined by Pierce BCA test). GSHred was assayed by monitoring NADPH oxidation at 340 nm during reduction of GSSG to GSH (32). Specific activity was expressed in μmol/L NADPH oxidized/min/mg of protein. γGCS activity was determined by monitoring formation of γ-glutamyl cysteine-γ-aminobutyrate in a coupled assay with pyruvate kinase and lactate dehydrogenase (33). GSH S-T was determined as outlined by Habig et al. (34). The change in absorbance at 343 nm was monitored in a solution of 1 mmol/L GSH, 1 mmol/L 1-chloro-2,4-dinotrobenzene (CDNB) in 0.1 mol/L phosphate buffer, pH 6.5, upon addition of supernatant, with specific activity expressed in μ H_2O_2 pyridine nucleotide or CDNB converted per min/mg of protein.

Statistical methods

Enzyme specific activities were compared for statistical significance using the unpaired Student's *t* test. Data presented in Figures 2 and 4–8 was subjected to statistical analysis using analysis of variance (ANOVA). Post hoc comparisons were conducted using Tukey's multiple comparison procedure to control for type I error. All tests for significance were conducted at the 0.05 significance level (SAS PROC GLM; SAS Institute Inc., Cary, NC, U.S.A.).

RESULTS

Cisplatin exposure causes a dose-related destruction of hair cells in vitro

Cisplatin exposure caused dose-dependent destruction of auditory hair cells in the organ of Corti explants (Figs. 2 and 3). Hair cell destruction began in the basal turns with lower doses of cisplatin and proceeded to the apex with increasing dosage. OHCs were destroyed at lower dosages than were IHCs. This pattern of hair cell loss is similar to what has been reported in vivo (8–12). Figure 3A illustrates the normal pattern of stereociliary bundles of the midturn area of an FITC-phalloidin–stained organ of Corti explant grown for 3 days in control medium. Three rows of outer and a single row of IHCs are present showing the normal V shape and linear organization in outer and IHC stereocilia, respectively. Exposure to cisplatin disrupted this normal pattern of FITC-phalloidin staining of the stereociliary bundles in the organ of Corti's explants (compare Fig. 3A and 3B). A few hair cells with intact cuticular plates were left in the cisplatin-exposed explants, but they had lost their normal pattern of organization and there were no stereocilia bundles present. Figure 2 graphically presents the mean hair cell density of organ of Corti explants as a function of cisplatin concentration and shows that the explants fall into two groups, with light to moderate hair cell loss occurring in the 4–6 μg/ml range of cisplatin in contrast to the heavy loss of hair cells that occurs in the 8–12 μg/ml concentration range of this neurotoxic compound. To contrast the results of the smallest to the largest dosage of cisplatin tested in our cultures, we observed that even at the lowest concentration (i.e., 4 μg) there was some hair cell destruction, whereas at the concentration of 12 μg/ml, < 30% of the original population of hair cells remained.

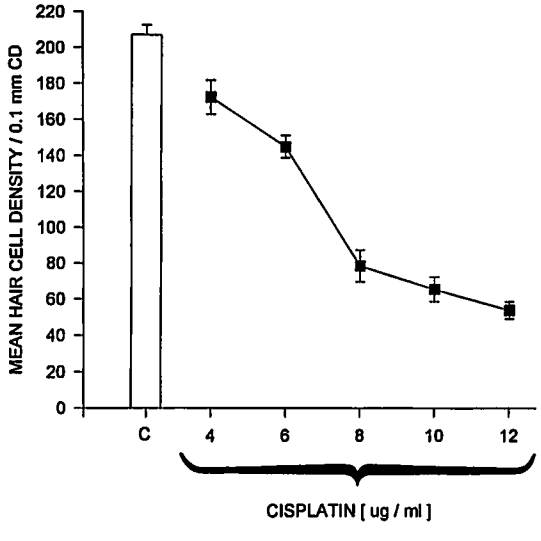

FIG. 2. A dose response determination of cisplatin toxicity on P-3 rat organ of Corti explants after 48 h of cisplatin exposure. Values represent mean hair cell density as determined by cuticular plate counts. Error bars define the standard error, and C represents control explant counts. Cisplatin was tested at a concentration range of 4–12 μg/ml.

Protectant molecules prevent cisplatin cochlear toxicity in vitro

Figure 4 represents the mean hair cell density of cisplatin-exposed (10 μg/ml) organ of Corti explants as a function of the protective agent tested. EBS with L-NAC had an additive effect on mean hair cell density of cisplatin-exposed explants that approached the hair cell density levels seen in control explants (Fig. 4).

D-MET and MTBA at their highest concentrations tested, as well as the combination of EBS plus L-NAC, afforded nearly complete protection of auditory hair cells in the cisplatin-exposed organ of Corti explants. ANOVA was conducted to test for effect of different treatments and concentrations of protectant molecules. There was a significant effect of treatment type ($F = 131.43$, $p < 0.001$). Post hoc comparisons (Tukey procedure) showed all treatments assayed and presented in Fig. 4, to be significantly better than untreated cisplatin-exposed explants. EBS plus L-NAC was better than either L-NAC or EBS alone. Hair cell densities of explants treated with D-MET at both concentrations tested, MTBA at the highest concentration tested (i.e., 10^{-3} mol/L), and the combination of EBS and L-NAC were not significantly different from the hair cell density of control explants. However, explants treated with MTBA 10^{-4} mol/L, EBS alone, and L-NAC alone were all significantly less than control levels of hair cell density, but were significantly better than the hair cell density of untreated, cisplatin-exposed organ of Corti explants. Figure 3C and 3D shows the appearance of an FITC-phalloidin–stained midturn section of organ of Corti explant exposed to cisplatin (10 μg/ml) but protected with D-MET at 10^{-2} mol/L (Fig. 8C) and 10^{-3} mol/L (Fig. 8D) concentrations. At the

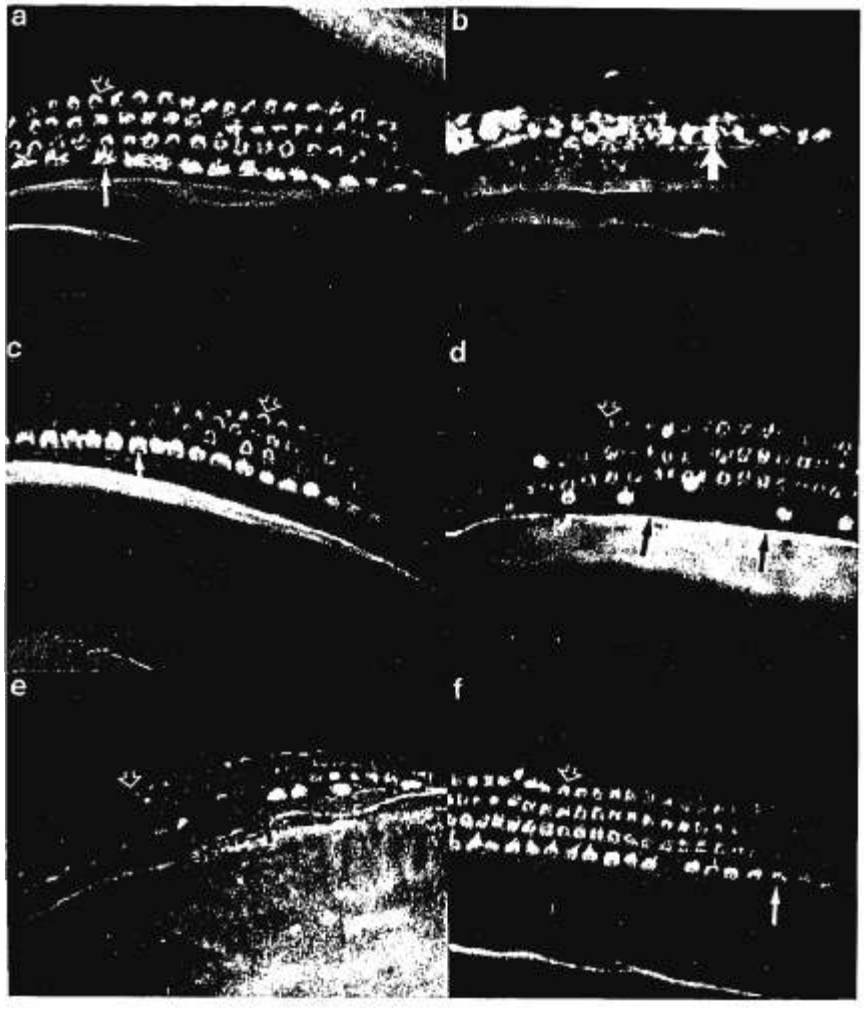

FIG. 3. P-3 rat organ of Corti explants after 72 h in vitro. FITC-phalloidin– stained stereocilia bundles and cuticular plates of whole-mount organotypic cultures viewed with a Zeiss Axiophot epifluorescent microscope. **A:** A control explant demonstrating orderly row of IHCs with organized stereocilia bundles (*solid arrow*) and three rows of OHCs with V-shaped stereocilia bundles (*open arrow*). **B:** An explant exposed to cisplatin (10 μg/ml), demonstrating a loss of a majority of its hair cells, a disrupted pattern of cytoarchitecture, and a loss of all stereocilia bundles. Arrow points to the cuticular plate of a remaining hair cell. **C:** An explant exposed to cisplatin plus D-MET 10^{-2} mol/L. Three orderly rows of OHCs are evident, as are a row of IHCs. The stereocilia bundles of both the inner (*solid arrow*) and OHCs (*open arrow*) appear to have a normal morphology. **D:** An explant exposed to cisplatin plus D-MET (10^{-3} mol/L). Overall cytoarchitecture has remained organized, but IHCs are missing from some segments of the cochlear duct, as indicated by the area bracketed by the black arrows. The open arrow points to an OHC with fused and missing stereocilia. Otoprotection by D-MET at this concentration is reduced compared with the higher concentration (i.e., 10^{-2} mol/L) seen in the photomicrograph of Fig. 2C. **E:** In an explant exposed to cisplatin plus L-NAC, 10^{-2} mol/L, three rows of normal appearing OHCs are evident. A row of IHCs shows some areas with missing hair cells as indicated by the black arrows. The open arrow points to an OHC that has lost its stereocilia bundle. **F:** An explant exposed to cisplatin plus L-NAC, 10^{-2} mol/L plus RPIA (100 μmol/L). Note the intact orderly rows of inner and OHCs with normal IHC stereocilia bundles (open and *solid arrows*, respectively).

higher concentration of D-MET, the hair cell density and integrity of stereocilia bundle morphology were similar to that observed and quantified (Fig. 4) for control explants. At the lower dose of D-MET there was some loss of stereocilia bundle integrity compared with control cultures (compare Fig. 3C and 3D; Fig. 5). As seen in Fig. 5, explants exposed to cisplatin only had no inner or OHC stereocilia bundles remaining after 72 h in vitro. Both D-MET and MTBA (10^{-3} mol/L) afforded significant protection to the stereocilia bundle integrity of IHCs exposed to cisplatin, whereas D-MET (10^{-2} mol/L) also provided complete protection of the integrity of OHC stereociliary bundles. EBS and EBS plus L-NAC were ineffective in preserving stereocilia bundle morphology for both inner and OHCs in these organ of Corti explants exposed to a 10 μg/ml concentration of cisplatin (data not shown). ANOVA demonstrated significant differences between treatment groups (IHC groups $F = 73.7$, $p < 0.001$; OHC groups $F = 33.82$, $p < 0.001$) and concentrations of protectant molecules (IHC groups $F = 262.52$, $p < 0.001$; OHC groups $F = 1,243$, $p < 0.001$). Post hoc analysis (Tukey) confirmed significance at the 0.05 confidence level.

BSO, L-NAC, RPIA, and GSH compounds reveal potential mechanisms of cisplatin toxicity

BSO is a specific inhibitor of the rate-limiting enzyme for GSH biosynthesis, γGCS (23). BSO has been demonstrated to reliably inhibit γGCS and to produce lower levels of intracellular GSH in a variety of cells and tissues both in vitro and in vivo (23,35–37).

If reduced GSH is important in the protection of hair cells from cisplatin toxicity, addition of BSO to organ of Corti explants should enhance their susceptibility to cisplatin toxicity. As seen in Fig. 6, BSO added to the culture medium at a dosage of 5 μmol/L 24 h before and then during the addition of cisplatin significantly shifted the dose response curve to the left. At the lower concentrations of cisplatin (i.e., 4-6 μg/ml), the differential effect of the BSO was greater than for the higher levels of

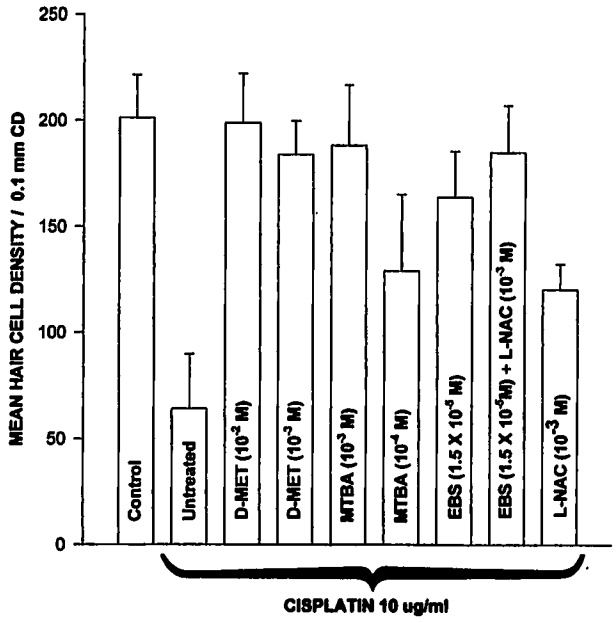

FIG. 4. Effectiveness of otoprotective agents in preventing cisplatin toxicity in P-3 rat organ of Corti explants. The concentrations of the protectant molecules are indicated within the graph bars. The cisplatin concentration was 10 μg/ml in all cultures, with the exception of the control explants. Error bars represent standard deviation. All of the otoprotective agents tested afforded highly significant protection from cisplatin toxicity as measured by hair cell density. D-MET, 10^{-2} and 10^{-3} mol/L), MTBA (10^{-3} mol/L), and EBS (1.5×10^{-5} mol/L) plus L-NAC (10^{-3} mol/L) produced near complete protection of the explants hair cells from cisplatin toxicity.

cisplatin (i.e., 8–12 μg/ml). Organ of Corti explants cultured for 72 h in BSO alone (5 μmol/L) had no abnormalities, i.e., no loss of stereocilia bundle integrity or hair cell death (data not shown). L-NAC is a thiol compound that can both act as a direct free radical scavenger and increase intracellular GSH levels both in vivo and in vitro (38–40). Addition of L-NAC to the medium for 24 h before and during the addition of cisplatin (10 μg/ml; 48 h) provided significant protection against cisplatin toxicity at concentrations of 6–12 μg/ml of this ototoxin (Fig. 6). RPIA is an adenosine agonist that has been found to rapidly stimulate the activity of a number of antioxidant enzymes in a variety of cell types in vitro (41) and in vivo (42). If antioxidant enzyme activity is important in preventing cisplatin-induced hair cell loss, then the addition of RPIA to explants should augment the effect of otoprotectant molecules. The combination of RPIA with L-NAC produced a significant shift to the right (i.e., an increase) of the otoprotective effect of L-NAC for all of the concentrations of cisplatin studied, i.e., 4–12 μg/ml (Fig. 6). Mean hair cell density in the L-NAC plus RPIA explants was at control levels even at the highest cisplatin concentration, i.e., 12 μg/ml. Interestingly, RPIA added alone at the same concentration afforded no protective effect for hair cells in cisplatin-exposed organ of Corti explants (data not shown). The data presented in Figure 6 were tested using ANOVA to assess effects of the various treatments and concentration. There was a significant effect for both the type of treatment ($F = 292.8$, $p < 0.001$) and the concentration of cisplatin ($F = 39.58$, $p < 0.001$). Post hoc comparisons (Tukey) clarified treatment differences at the 0.05 significance level. The data presented in Fig. 7 demonstrates that L-NAC was effective in protecting the integrity of IHC stereocilia at all but the highest cisplatin concentration tested (i.e., 12 μg/ml). The combination of L-NAC and RPIA augmented the protective effect of L-NAC so that complete protection of IHC stereocilia bundles was achieved even at the cisplatin concentration of 12 μg/ml. At all cisplatin concentrations tested, the combination of L-NAC plus RPIA maintained OHC stereocilia bundle integrity at control explant levels, achieving significantly better protection than afforded by L-NAC alone (compare Fig. 3E and 3F; Fig. 7). However, L-NAC by itself did afford significant protection of OHC stereocilia bundle integrity over cisplatin-exposed untreated explants (Figs. 3E and 7). Figure 3E and F portray the FITC-phalloidin–stained appearance of the midturn of cisplatin-exposed explants protected with either L-NAC (10^{-2} mol/L) (Fig. 3E) or a combination L-NAC (10^{-2} mol/L) plus RPIA (100 μmol/L) (Fig. 3F). A comparison of the appearance of these protected explants (Fig. 3E and F) to a cisplatin only exposed explant (Fig. 3B) readily shows that significant preservation of hair cell density and cochlear duct cytoarchitecture has been achieved by both of these protection treatment regimes. In contrast to both cisplatin only (Fig. 3B) and cisplatin plus L-NAC (Fig. 3E), the combination of L-NAC plus RPIA with cisplatin exposure (Fig. 3F) shows a near control level hair cell density and intact inner and OHC stereocilia bundle morphology. ANOVA of the data in Figure 7 showed significant differences for treatment type (IHC, $F = 36.62$, $p < 0.001$; OHC, $F = 111.16$, $p < 0.001$). Tukey analysis confirmed significant differences between each pair of treatment conditions for both inner and OHCs.

The effectiveness of L-NAC, a potential source of cellular GSH, in protecting hair cells in vitro and the additional protective effect of RPIA, a known stimulator of antioxidant enzyme activity, suggest that GSH may be important in the hair cells' defense against cisplatin toxicity (Figs. 3, 6, and 7). We assessed the ability of GSH and GSHe to prevent cisplatin-induced hair cell damage/destruction in organ of Corti explants (Fig. 8). GSH esters such as GSHe are more readily taken up into cells and afford higher levels of intracellular GSH after deesterification than does treatment with nonesterfied GSH (28,35,43). The effectiveness of treatment with GSHe over GSH in preventing auditory hair cell destruction in the explants is shown in the hair cell density counts presented in Fig. 8. There was a dose-related preservation of auditory hair cell density at all concentrations tested. GSHe was significantly better than GSH as an otoprotectant in cisplatin-challenged (10 μg/ml) explants at all of the concentrations tested (ANOVA treatment, $F = 20.59$, $p < 0.001$; dosage, $F = 33.51$, $p < 0.001$;

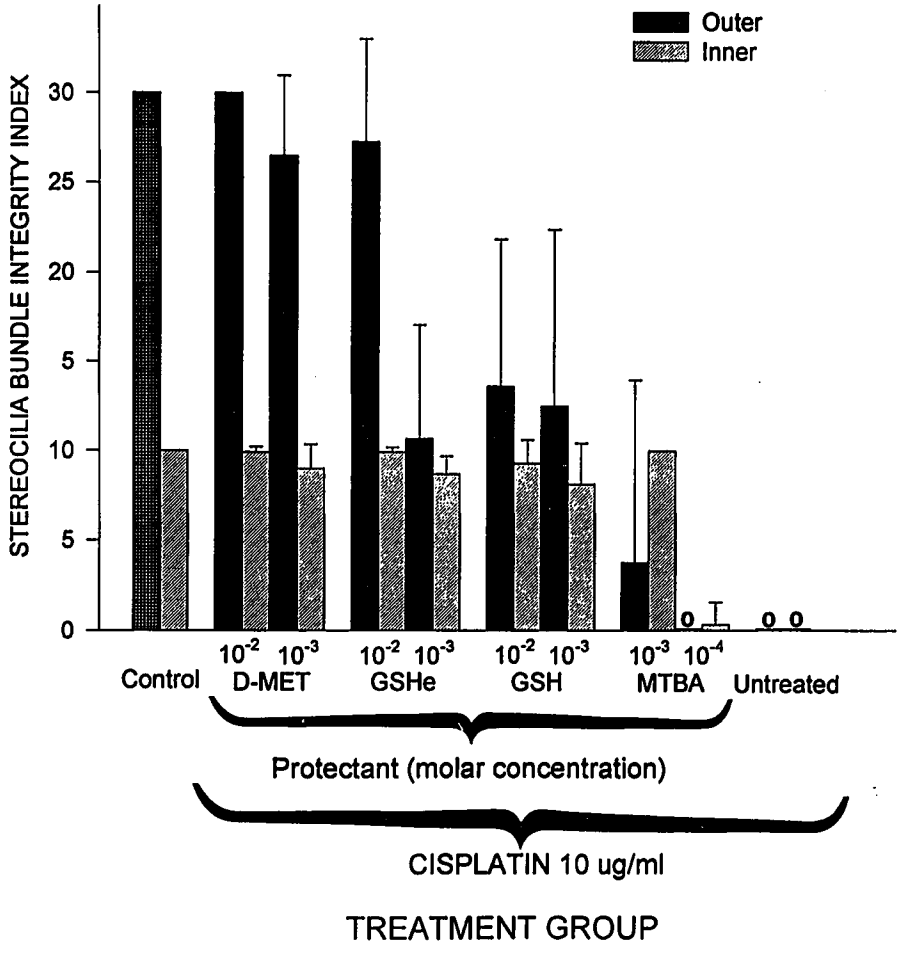

FIG. 5. Effect of otoprotectant molecules on the integrity of stereocilia bundle morphology in cisplatin-exposed P-3 rat organ of Corti explants. Explants were either maintained under control conditions or exposed to either cisplatin alone or cisplatin plus selected concentrations of protectant molecules. Cisplatin concentration was 10 μg/ml in all exposed cultures. Molar concentration of protectants is as indicated. Stereocilia bundle integrity index was determined by scoring stereocilia bundles as normal or disorganized (intact) or fused or absent (missing) for 10 IHCs and 30 OHCs for each cochlear turn and averaged. Error bars indicate standard deviation. IHC and OHC indices are indicated by the shading of the bars. D-MET (10^{-2} and 10^{-3} mol/L) and GSHe (10^{-2} mol/L) provided near complete protection of IHC and OHC stereocilia bundle morphology from cisplatin damage. GSHe (10^{-3} mol/L), GSH (10^{-2} and 10^{-3} mol/L), and MTBA (10^{-3} mol/L produced nearly complete protection of IHC stereocilia but less significant protection of OHC stereocilia bundle morphology. MTBA (10^{-4} mol/L) did not afford any significant protection of stereocilia bundle morphology.

all pairwise comparisons between treatments at each dosage level were significant by Tukey tests). GSHe was also significantly more effective than GSH at preserving the integrity of the OHC stereocilia bundles at the concentration of 10^{-2} mol/L (Fig. 5).

The data presented thus far support the hypothesis that exposure to cisplatin leads to hair cell damage through the generation of ROS that damage the hair cells and their stereocilia. The data also support the idea that bolstering the antioxidant defense system by increasing intracellular levels of GSH provides protection of auditory hair cells from destruction by ROS.

Real-time confocal analyses of ROS and GSH in live explants

Real-time confocal images of auditory hair cells in control explants were incubated with a mixture of calcein blue and DCF (Fig. 9A and B, respectively). The calcein blue fluorescent staining (Fig. 9A) delineates a row of hair cells as seen in cross-section (Fig. 9A). Control hair cells are devoid of DCF fluorescence (Fig. 9B), and the bright images of hair cells in Fig. 9A appear as dark images of those same hair cells. Figure 9D and E represents confocal images of hair cells exposed for 12 h to cisplatin (10 μg/ml). Hair cells within the cisplatin-exposed explants also can be identified by bright fluorescent staining with calcein blue (Fig. 9D). In contrast to control explants after cisplatin exposure, explants now show a bright DCF fluorescent staining of the majority of the hair cells (Fig. 9E), indicating the presence of H_2O_2 (ROS) within these hair cells. Figure 9G and H represent images of hair cells exposed to cisplatin (10 μg/ml) plus the otoprotectant molecule GSHe for 12 h. Hair cells in these GSHe-protected cisplatin-exposed explants are again easily identified by the calcein blue fluorescent staining (Fig. 9G), but in contrast to Fig. 9E (i.e., DCF staining of cisplatin only explants), the hair cells in the protected explants (Fig. 9H) demonstrated almost no DCF fluorescence, similar to the level of DCF fluorescence observed in the control explants (compare Fig. 9B and 9H). Thus, GSHe has effectively blocked the generation of ROS in the hair cells of cisplatin-exposed explants. In these experiments, bright DCF fluorescence was seen as early as 2 hours after cisplatin exposure, and hair cells remained brightly stained for the presence of ROS until hair cell integrity was lost 24–36 hours after cisplatin exposure. GSHe completely prevented the DCF florescence at all time points, that is, 2–36 hours.

Figure 9C, F, and I represents real-time confocal images of hair cells from organ of Corti explants incubated

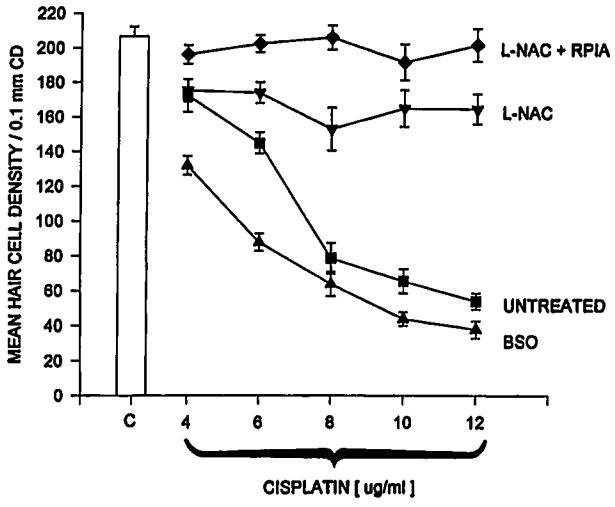

FIG. 6. Otoprotective effect of L-NAC and L-NAC plus RPIA, and deleterious effect of blocking GSH synthesis (BSO) on cisplatin toxicity. Cisplatin dose response curves (4–12 μg/ml) are presented as mean hair cell density per 0.1 mm of cochlear duct length. Explants were exposed to either cisplatin plus BSO (5 μmol/L), cisplatin plus L-NAC (10^{-2} mol/L), cisplatin plus L-NAC (10^{-2} mol/L) plus RPIA (100 μmol/L), or cisplatin only (untreated). Open bar indicates hair cell density of control explants (C, not exposed to cisplatin). After an initial 24 h in vitro, explants were exposed to cisplatin for a period of 48 h. Explants treated with BSO, L-NAC, or L-NAC plus RPIA received these compounds during the initial 24 h in vitro (pretreatment period) and again during the following 48 h of cisplatin exposure. Bars indicate standard error of means. BSO treatment shifted the dose response curve for cisplatin exposure significantly to the left (increased damage), with the greatest effect seen at the lower levels of cisplatin exposure (i.e., 4–6 μg/ml). L-NAC provided good protection for hair cells from cisplatin toxicity at all of the concentrations tested, and the combination of RPIA with L-NAC resulted in near complete protection of hair cells in the explants at all of the cisplatin concentrations tested.

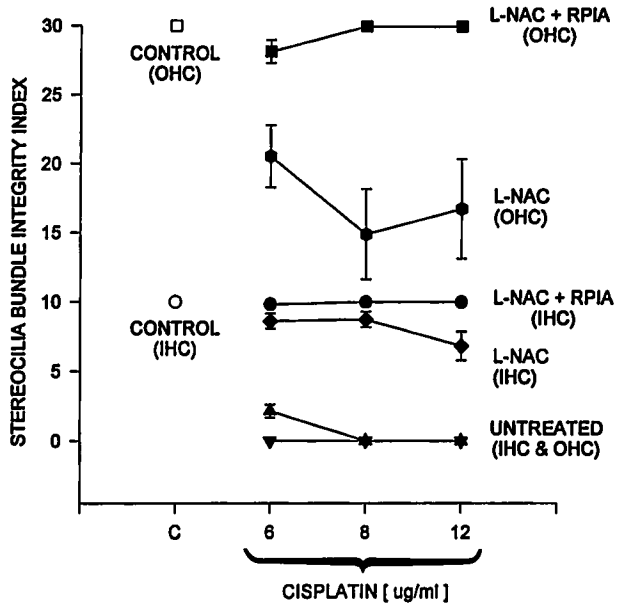

FIG. 7. Effect of L-NAC and RPIA on the preservation of stereocilia bundles in cisplatin-exposed P-3 rat organ of Corti explants. Explants were exposed to varying concentrations (6–12 μg/ml) of either cisplatin alone, cisplatin plus L-NAC (10^{-2} mol/L), or cisplatin plus L-NAC (10^{-2} mol/L) plus RPIA (100 μmol/L). Stereocilia bundle integrity index was determined by direct scoring of stereocilia bundle morphology and compared with control explant stereocilia bundle integrity. IHC stereocilia bundle integrity index (○) and OHC stereocilia bundle integrity index (□) are indicated for control conditions. Cisplatin exposure (untreated) eliminated almost all stereocilia bundles at a dose level of 6 μg/ml and destroyed all stereocilia bundle integrity at both 8 and 12 μg/ml exposure levels. L-NAC and L-NAC plus RPIA equally preserved IHC stereocilia integrity at 6 and 8 μg/ml exposure levels, whereas only L-NAC plus RPIA afforded complete IHC protection at the highest cisplatin concentration tested (i.e., 12 μg/ml). L-NAC significantly enhanced OHC bundle integrity, but the combination of L-NAC plus RPIA brought the OHC stereocilia bundle integrity index to control levels. Error bars represent standard error of means.

with MCB to detect the intracellular presence of reduced GSH. Figure 9C is an image from a control explant demonstrating bright intracellular MCB fluorescent staining of hair cells in Corti's organ, in contrast to Fig. 9F, which shows a marked reduction in MCB fluorescent staining of the hair cells within a cisplatin-exposed explant. The presence of GSHe during cisplatin exposure results in MCB levels of fluorescent staining of hair cells that are at approximately the same intensity of staining observed in control explants (compare Fig. 9C and 9I). In this experiment, MCB fluorescent staining of hair cells in cisplatin-exposed cochleae appeared to initially increase and then began decreasing at 12 h, with a further decrease in stain intensity at the 24-h post–cisplatin exposure time point. In GSHe-protected cisplatin-exposed explants, MCB fluorescence remained at control levels at all of the time points studied, i.e., 2–36 h.

Confocal experiments with MCB staining were also performed to assess the effect of BSO and the combination of BSO plus cisplatin on hair cell GSH levels. Figure 10 displays the MCB fluorescent stain confocal images of hair cells from control, BSO treated, and BSO treated plus cisplatin-exposed organ of Corti explants after 12 h in vitro. As can be seen in Figure 10, exposure of explants to BSO dramatically reduced MCB fluorescent staining of hair cells as compared with levels of staining present in control explants. To determine if exposure to a combination of BSO-cisplatin would further reduce MCB fluorescent staining of hair cells, the sensitivity of the confocal image analysis was increased 18-fold. The very faint image seen in Fig. 10B now appears bright as depicted in Fig. 10C. This now allows visualization of the further reduction in the MCB fluorescent staining of hair cells in BSO-treated explants that have also been exposed to cisplatin (10 μg/ml) (compare Fig. 10D and 10C.

Effects of cisplatin and RPIA on antioxidant enzyme levels

Cisplatin is known to bind to thiol groups on a variety of cellular proteins, inactivating them. Inactivation of cochlear GSH peroxidase and reductase (20,21) and kid-

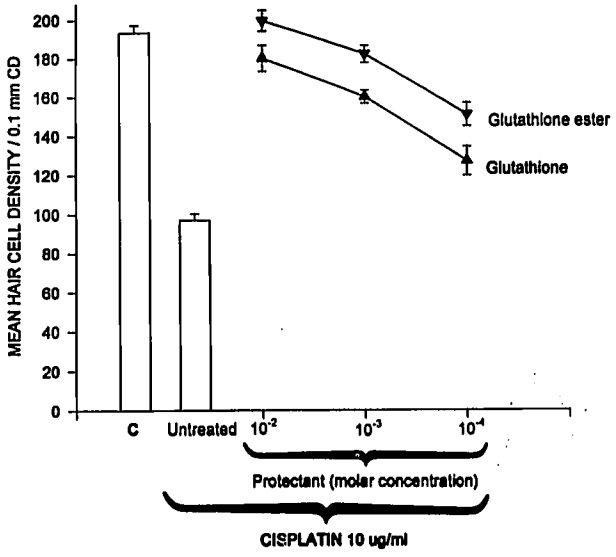

FIG. 8. Effect of GSH and GSHe on protection of the hair cells from cisplatin toxicity in cisplatin exposed P-3 rat organ of Corti explants after 72 h in vitro. Explants were cultured under control conditions or exposed to cisplatin 10 μg/ml (untreated) or exposed to cisplatin (10 μg/ml) plus either reduced GSH or GSHe at the indicated concentrations. The mean hair cell density of control (C) and (cisplatin-exposed) untreated explants are indicated by open bars. Error bars indicate standard error of means. Reduced GSH and glutathione ester both afforded significant protection as measured by mean hair cell density at all of the concentrations tested. Glutathione ester provided significantly greater protection of the hair cells from cisplatin toxicity than GSH.

ney GSH S-T (44) by cisplatin has been observed. RPIA is an adenosine antagonist that acts through adenosine receptors to upregulate antioxidant enzyme activity in a number of cell types (41). RPIA placed on the round window membrane of a chinchilla also significantly elevated cochlear antioxidant enzyme activity for superoxide dismutase and GSH peroxidase (42).

We assayed the activity of four antioxidant enzymes (i.e., catalase, GSHred, γGCS, and GSH S-T) in the organ of Corti explant tissues. After an initial 24 h in vitro, explants were grouped into three exposure conditions: control, cisplatin (10 μg/ml), or RPIA (100 μmol/L) for the second 24-h period. After 48 h in vitro, explants were frozen at −70°C and analyzed for enzyme activity. As shown in Figure 11 when compared with control condition explants, there was a significant increase in the activity of catalase (30%), γGCS (30%), and GSH S-T (10%) activity in explants exposed to RPIA. Exposure to cisplatin, on the other hand, had no significant effect on the activity levels of these enzymes when compared with control explant enzyme levels. Exposure to cisplatin for 48 h (72 h in vitro) produced morphologic evidence of damage or cell death in the majority of hair cells. Reduction of GSH S-T activity to nondetectable levels, and 30% and 35% reduction of GSHred and catalase activities (data not shown) may represent an effect of cell death and may be far downstream of any initial cisplatin effects.

DISCUSSION

This study demonstrates the utility of P-3 rat organ of Corti explants as an in vitro model for the study of cisplatin auditory hair cell toxicity. Similar in vitro models have been reported to assess aminoglycoside toxicity in cochlear cultures (45,46) and cultures of vestibular receptors (47,48). Malgrange et al. have used neonatal rat organ of Corti explants to study cisplatin ototoxicity (personal communication). In the present study, organ of Corti explants cultured under control conditions reproducibly maintained both hair cell density and stereocilia bundle integrity (Figs. 2, 4, and 8). As reported in previous in vivo studies (8–12), the results of this study showed that as concentrations of cisplatin increased, the loss of hair cells and stereocilia bundle integrity began with the third row of OHCs and progressed to the first row of OHCs and then to the IHCs. There was also a base-to-apex pattern of progression of cisplatin toxicity in these explants. The similarities between published (8–12) in vivo cisplatin effects on hair cells and the findings of this study suggest that the cisplatin concentrations used in this in vitro study are physiologically relevant. One potential limitation of this in vitro model was that retinoic acid stimulates immature cochlear tissue (i.e., P-3 rat organ of Corti explants) and may respond differently to ototoxins than tissue from more mature animals (49).

The data obtained from the present study contribute to a further understanding of the mechanisms of cisplatin toxicity on auditory hair cells. The confocal imaging results suggest that cisplatin exerts its toxicity in hair cells by generating ROS and depleting intracellular stores of GSH (Fig. 9). GSH depletion by itself probably is not a sufficient insult to damage auditory hair cells because in the present study significant GSH depletion by exposure of explants to 5 μmol/L BSO was not associated with any morphologic evidence of hair cell loss or damage (Figs. 6 and 10). Consistent with the present study, ROS generation and GSH depletion have been implicated in cisplatin nephrotoxicity, whereas GSH, L-NAC, and the antioxidant enzyme superoxide dismutase have been found to be nephroprotective (15–19). Also, Ravi et al. found that whole cochlear GSH, GSHred, and GSH peroxidase levels decreased and malondialdehyde (a marker of lipid peroxidation) levels increased when rats were acutely exposed to a high-dose level of cisplatin (21). Rybak et al. (20) found that cisplatin-induced GSH depletion and GSH peroxidase inactivation in cochlear tissues could be prevented by pretreatment with a thiol containing compound, i.e., diethyldithiocarbamate. These in vivo studies (21,22) are consistent with the in vitro findings of the present study, in which thiol-containing compounds also afforded protection against cisplatin toxicity (Figs. 3–9).

In this study, L-NAC alone or in combination with EBS or RPIA conferred significant protection of auditory hair cells from cisplatin toxicity (Figs. 4, 6, and 7). Exogenous L-NAC can serve as a source of intracellular cysteine and increase intracellular GSH levels (50). The protection afforded by L-NAC is reported to result from

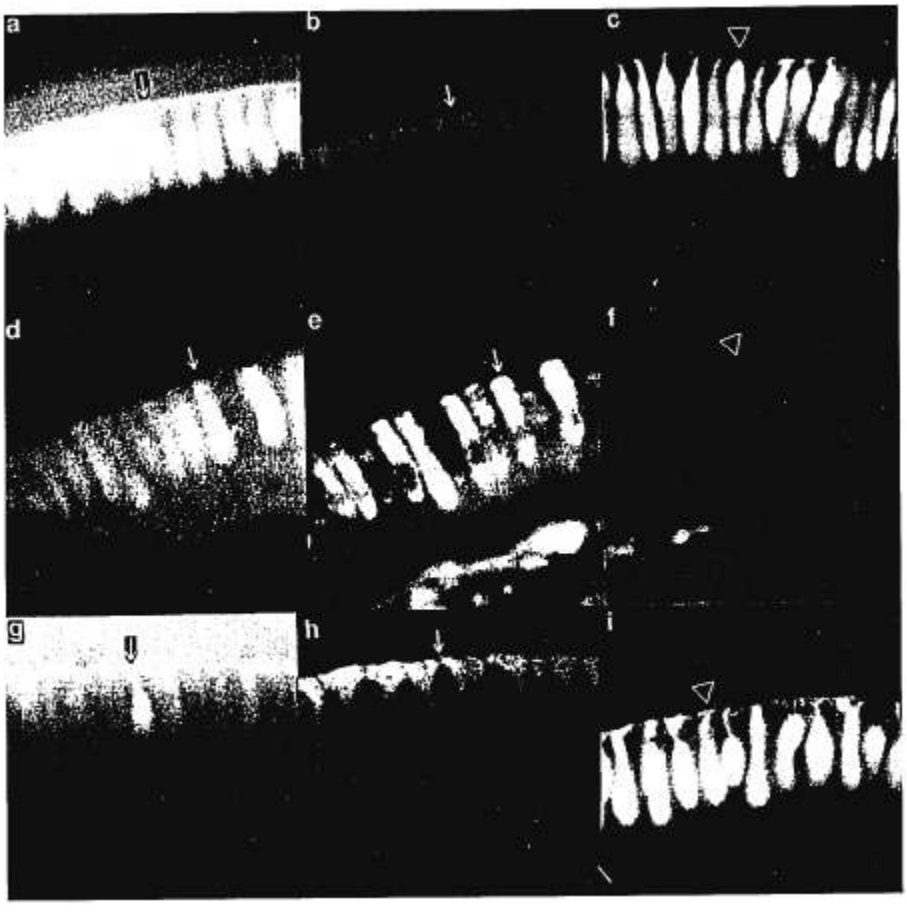

FIG. 9. Real-time confocal analysis of ROS formation and GSH depletion after either control conditions, cisplatin exposure, or otoprotectant plus cisplatin exposure in P-3 rat organ of Corti explants. All images represent midturn section of organ of Corti explants after 12 h in vitro. The confocal photomicrographs in **A, D,** and **G** represent imaging with the calcein blue AM chromophore (defines hair cells) under control (**A**); cisplatin, 10 μg/ml (**D**); and cisplatin 10 μg/ml plus GSHe 10^{-2} mol/L (**G**) conditions. **B, E,** and **H:** DCF chromophore (detects ROS) imaging under control (**B**), cisplatin 10 μg/ml (**E**) and cisplatin 10 μg/ml plus GSHe 10^{-2} mol/L (**H**) conditions. **C, F,** and **I:** MCB (detects GSH) under control (**C**), cisplatin 10 μg/ml (**F**), and cisplatin 10 μg/ml plus GSHe, 10^{-2} mol/L (**I**) conditions. DCF and MCB excitation and photographic parameters were kept constant between different specimens. Calcein blue AM images and DCF images are of identical hair cells at two different wavelengths to take advantage of double labeling of the same hair cell. The arrows point to identical hair cells incubated with calcein blue AM plus DCF (compare A and B; D and E; and G and H). Open arrowheads in C, F, and I point to hair cells incubated with MCB. Images of control samples show bright fluorescence in hair cells with calcein blue AM (**A**). Control explant hair cells, as expected, were devoid of DCF fluorescence (**B**) and brightly fluorescent with MCB (**C**). Images of cisplatin-exposed explants again demonstrate fluorescing hair cells with calcein blue AM (**D**). These same hair cells now brightly fluoresce for the presence of activated DCF (**E**) indicating the presence of ROS. In the cisplatin-exposed explants, the MCB fluorescence of the hair cells now is greatly diminished (**F**) as compared with that seen in control explants (**C**). Hair cells exposed to cisplatin plus GSHe (otoprotectant) again are visible with calcein blue AM staining (**G**). Hair cells protected from cisplatin toxicity by GSHe demonstrate reduced DCF fluorescence (**H**) similar to the level of fluorescence seen in the hair cells of the control explants (**B**). GSHe-protected hair cells also demonstrate MCB fluorescence (**I**) similar to the levels seen in control explants (**C**) and much brighter than the levels seen in the hair cells of the unprotected cisplatin exposed explants (**F**).

its ability to increase cellular cysteine and GSH and/or to scavenge ROS (51). L-NAC also can serve as a cosubstrate for EBS because it functions as a peroxidase mimic (52) and EBS activity is increased in the presence of thiol-containing compounds such as L-NAC (53). This is consistent with our findings of protection from cisplatin-induced toxicity with L-NAC and L-NAC plus EBS (Fig. 4). RPIA has been noted to increase antioxidant enzyme activity of a variety of cell types in vitro (41) and in whole cochlea in vivo (42). We found that exposure of the explants to RPIA for 24 h led to a significant increase in catalase, γGCS, and GSH S-T activity (Fig. 11). RPIA, in the presence of L-NAC, was able to confer additional preservation of hair cell density and OHC stereocilia morphology (Fig. 7). One explanation for this finding could be that L-NAC increases intracellular cysteine, which then becomes a substrate for γGCS for restoring intracellular GSH levels (23,50).

We found that both GSH and GSHe preserved hair cell density and stereocilia bundle integrity and that GSHe was more effective than GSH in this regard (Fig. 8). GSHe also prevented ROS production and GSH depletion induced by cisplatin, as seen in the confocal study (Fig. 9). This is consistent with data of Anderson et al. (43), showing a nephroprotective effect of GSH and GSH monoisopropyl ester. The ester was more effective than nonesterfied GSH, and this was thought to be due to an increase in the cellular uptake of the monoisopropyl ester (43), as has been shown for the diethyl ester of GSH (28). Malgrange also found that GSH protects the hair cells of organ of Corti explants from cisplatin toxicity (personal communication).

We have identified several protective agents that protected the hair cells in organ of Corti explants from cisplatin toxicity. Three of the thiol-containing compounds studied, i.e., D-MET, MTBA, and EBS, may protect by several mechanisms. Complexing cisplatin, reversing cisplatin complexes with cellular thiols, or scavenging ROS (e.g., H_2O_2) are all probabilities. All three of these compounds have been found to be nephroprotective from

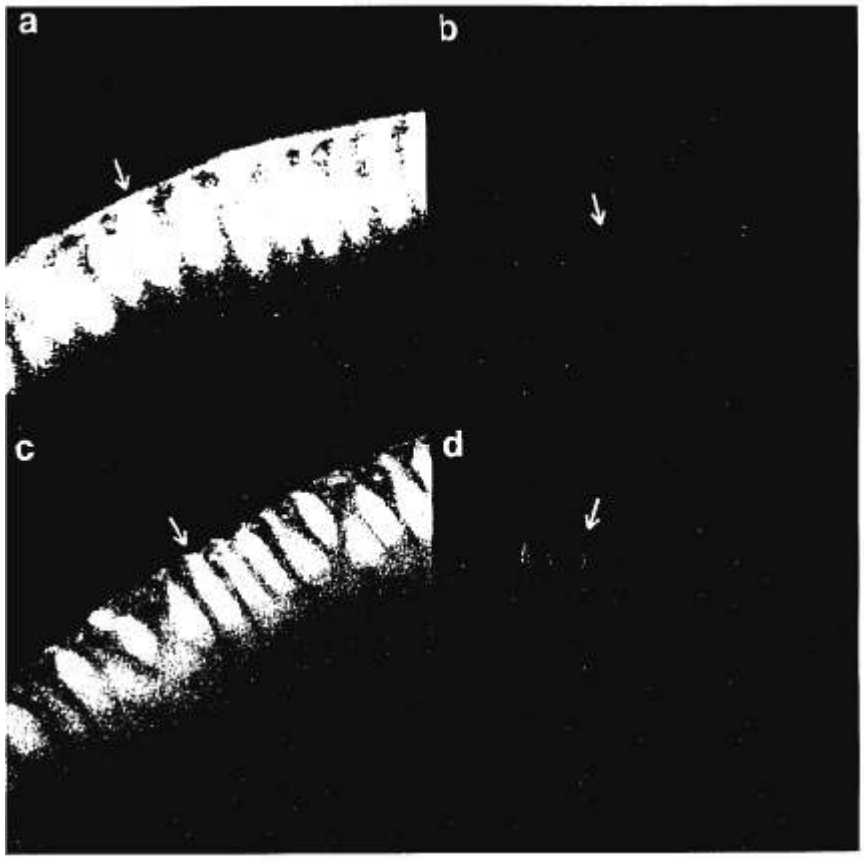

FIG. 10. Effect of BSO on intracellular levels of GSH in the hair cells of control and cisplatin-exposed P-3 rat organ of Corti explants. Cochlear explants were cultured under control conditions or cultured in the presence of 5 μmol/L BSO or 5 μmol/L BSO plus cisplatin 10 μg/ml, for a period of 12 h. Explants were then incubated with MCB for confocal imaging of levels of fluorescent stain intensity. MCB fluoresces in the presence of intracellular reduced GSH. The confocal images in **A** and **B** were made under identical intensity of laser illumination and with the same photographic parameters. A comparison of the MCB fluorescence in the hair cells of a control explant (**A**) to those of BSO treated explants (**B**) shows a marked decrease in fluorescent stain intensity within the hair cells as a result of BSO exposure, indicating a reduction in intracellular GSH levels. The confocal images in **C** and **D** were obtained by an 18-fold increase in the confocal laser light intensity. At an 18-fold increase in intensity, MCB fluorescence is now visible in hair cells (**C**) of the BSO-exposed explant (i.e., 5 μmol/L for 12 hours). Hair cells exposed to both BSO 5 μmol/L and cisplatin 10 μg/ml (**D**) show an even further decline in MCB fluorescent stain intensity compared with explants treated with BSO alone, **C**). The images in **C** and **D** were obtained at the same laser intensity and using the same photographic parameters.

cisplatin toxicity in rodents (52–57). D-MET is the stereoisomer of L-methionine, an essential amino acid. D-MET may prevent cisplatin toxicity by binding to it and thereby preventing its toxic effects, by reversing the binding of cisplatin to GSH and protein thiols, or by protecting free L-methionine from cisplatin binding, thereby keeping it available for protein synthesis and cellular metabolism (56–58). MTBA through its thioether group can act as an ROS scavenger and also complex with cisplatin, minimizing its toxic effects (54). EBS is a selenoorganic compound that reduces damaging hydroperoxides at the expense of cellular thiols by mimicking the action of GSH peroxidase while using GSH, L-NAC, or other thiols as a cosubstrate (52,53,55). In a thiol-rich cellular environment, EBS forms selenols that can remove platinum from protein-thiol bonds and inactivate cisplatin (55). The increased protection seen in our study by the combination of L-NAC with EBS versus EBS alone would be consistent with the previously mentioned published data.

Importantly, the in vitro protectants D-MET, MTBA, and EBS also have been shown to protect against cisplatin ototoxicity in rats (59–61). Pretreatment of rats with one of the protectants prevented the acute auditory brain stem threshold shifts induced by cisplatin (59–61), and D-MET prevented hair cell destruction as seen by scanning electron microscopic analysis of cochlear tissue taken against cisplatin ototoxicity 72 h after cisplatin administration (59). Thus, there is a good correlation be-

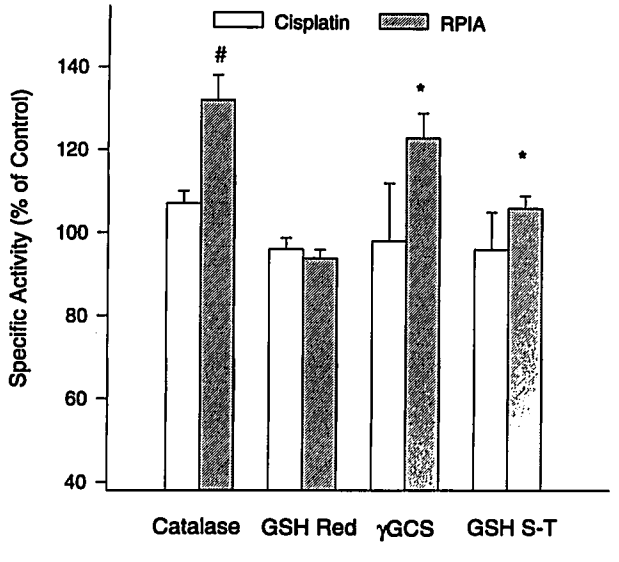

FIG. 11. Effect of cisplatin and RPIA on antioxidant enzyme activity in P-3 rat organ of Corti explants. Enzyme activity is expressed as a percentage of that determined for control explants for catalase, GSHred, γGCS, and GSH S-T. After 24 h of cisplatin exposure, there are no significant changes in the levels of the four enzymes assayed as compared with control explant levels. RPIA (100 μmol/L) exposure for a 24-h period, on the other hand, does cause a significant increase in the specific activity levels of catalase, γGCS, and GSH S-T (#$p < 0.001$, *$p < 0.02$, unpaired Student's *t* test).

tween the ability of protectants identified in vitro with their ability to also protect in vivo.

In summary, organotypic culture of Corti's organ can serve as a model system for studying cisplatin auditory hair cell toxicity. In our study (Fig. 1), we found direct evidence that cisplatin exposure is associated with the generation of ROS and depletion of GSH in auditory hair cells in our in vitro model (Fig. 9). Agents that augmented the antioxidant defense system (i.e., RPIA; Figs. 6, 7, and 11) prevented a decrease in hair cell density and preserved stereocilia bundle morphology. These effects correlated with in vivo protection of cochlear hair cells from cisplatin damage by agents identified as being protective in vitro. These identified protectant molecules, or others yet to be discovered, may one day prove to be clinically useful in limiting or completely preventing cisplatin ototoxicity.

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