

Direct detection of ototoxicant-induced reactive oxygen species generation in cochlear explants

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Received 23 July 1995; revised 17 April 1996; accepted 20 April 1996

Abstract

The proposal that free-radical generation contributes to the ototoxicities of several chemical agents was studied utilizing electron paramagnetic resonance (EPR) spectrometry to detect directly ototoxicant-induced reactive oxygen species formation in cochlear tissue. Guinea pig cochlear explants in chelexed artificial perilymph (AP; 200 μ l) were exposed to an ototoxicant or AP for 10 min. Ototoxic agents included gentamicin sulfate (4.0 mM), kanamycin monosulfate (4.0 mM), ethacrynic acid (0.5 mM), furosemide (0.3 mM), cisplatin (0.1 mM), trimethyltin chloride (0.1 mM), and quinine HCl (3.0 mM). Following incubation, 20 μ l of AP/ototoxicant mixture was replaced by the filtered spin trap, 5,5-dimethylpyrroline-*N*-oxide (DMPO). After 10 min, the EPR spectrum of the mixture was obtained. Four line EPR spectra of relative intensities 1:2:2:1, associated with hydroxyl radical (OH \cdot)/DMPO adduct formation, were evidenced by reaction mixtures containing cochlear explants exposed to each ototoxicant. Cisplatin, quinine and the loop diuretics produced weak OH \cdot -associated EPR signals in the absence of a cochlear explant, which were amplified in its presence. Deferoxamine quenched all OH \cdot spectral peaks. Peroxide levels, assayed in parallel experiments, were diminished by each ototoxicant relative to those seen following AP exposure, suggesting possible H₂O₂ conversion to OH \cdot . These data support the proposal that various ototoxic agents are capable of reactive oxygen species generation or promotion in cochlear tissues.

Keywords: Cochlea; Electron paramagnetic resonance spectrometry; Free radical; Ototoxicity; Reactive oxygen species

1. Introduction

Ototoxicants induce hearing loss by specifically disrupting cochlear function and include such structurally diverse chemical groups as the aminoglycoside antibiotics, loop diuretics, antimalarial agents and environmental toxicants (i.e., organic solvents and metals). Although the structural damage induced by such agents in the cochlea is well documented, their specific mechanisms of action within the cochlea remain poorly understood. At present, there are

no adequate theoretical frameworks for predicting the ototoxic potential of chemicals (Schacht, 1986) or for determining methods of preventing cochlear hearing loss of chemical origin. Speculations about the mechanisms of xenobiotic damage in other organ systems have recently focused upon three, non-exclusive processes: alterations in intracellular free Ca²⁺ (Nicotera et al., 1992), excitatory amino acid accumulation (Olney, 1990) and free-radical generation and related damage (Halliwell and Gutteridge, 1985; LeBel and Bondy, 1991).

Reactive oxygen species (ROS) are intermediates of aerobic metabolism. The superoxide anion (O₂⁻) is a free radical produced in the mitochondrial electron transport chain, which can be chemically or enzymatically dismutated to hydrogen peroxide (H₂O₂) plus O₂. H₂O₂ is not a true free radical but is a ROS capable of causing extensive cellular damage. O₂⁻ and H₂O₂ in the presence of a redox-sensitive transition metal, such as iron, can be converted via the Fenton reaction to form the hydroxyl radical (OH \cdot); O₂⁻ reduces Fe³⁺ to Fe²⁺, which in turn, reduces

Abbreviations: AP, artificial perilymph; DMPO, 5,5-dimethylpyrroline-*N*-oxide; EPR, electron paramagnetic resonance; GSH, glutathione; H₂O₂, hydrogen peroxide; IHC, cochlear inner hair cell; O₂⁻, superoxide anion; OH \cdot , hydroxyl radical; OHC, cochlear outer hair cell; ROS, reactive oxygen species; SEM, standard error of the mean; SGC₁, type I spiral ganglion cell

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H₂O₂ to produce OH[•] (Halliwell and Gutteridge, 1985; Halliwell, 1992). ROS induce cellular damage by causing DNA strand breaks, lipid and protein oxidation, perturbed membrane permeability and ionic transport, and the alteration of membrane-bound enzymes and receptors (Butterfield et al., 1994; Hensley et al., 1994; Hall et al., 1995). These changes may be indexed by increased levels of lactate dehydrogenase leakage and malondialdehyde formation, diminished levels of glutathione (GSH), and altered levels of antioxidant enzymes, such as, superoxide dismutase, catalase and GSH-peroxidase (Ravi et al., 1995; Rybak et al., 1995).

In the auditory system, recent circumstantial evidence has supported a role for ROS formation following exposure to the aminoglycoside antibiotics, loop diuretics and certain ototoxic metals (e.g., cisplatin, trimethyltin). Four lines of evidence serve as such support: (1) ROS scavengers frequently protect against the ototoxic effects of these agents (Mizukoshi and Asano, 1976; Pierson and Møller, 1981; Otto et al., 1988; Schweitzer, 1993; Garetz et al., 1994a; Clerici, 1996); (2) agents which disrupt the GSH reducing system potentiate the ototoxicity of some such agents (Hoffman et al., 1988); (3) exposure of isolated outer hair cells (OHC) to conditions that generate ROS in other tissues, causes structural damage for which ROS scavengers provide protection (Mizukoshi and Asano, 1976; Clerici et al., 1995a; Dulon et al., 1989; Garetz et al., 1994b); and (4) some of these agents alter cochlear biochemical indices associated with oxidative damage (Ravi et al., 1995; Rybak et al., 1995).

However, no direct determination of ROS generation by cochlear tissues following ototoxicant exposure has yet been reported. Electron paramagnetic resonance (EPR) spectrometry is the only direct method of detecting oxygen- and carbon-centered radicals generated in tissues and fluids. In EPR spin trapping studies, a non-paramagnetic nitron (the trap) reacts with a transient free radical (the spin) to produce a paramagnetic spin adduct, detectable spectrometrically. In order to determine whether any of the classic categories of ototoxicants can induce the formation of O₂⁻ or OH[•] in cochlear tissue, cochlear explants in primary culture were exposed to candidate agents of various ototoxicant categories in the presence of a spin trapping agent. The PeroXOquantTM assay was utilized as a confirmatory measure, to index peroxide formation. Results indicate that metal catalyzed OH[•] formation is a common outcome of ototoxicant exposure under these conditions. A preliminary report of these findings has been presented (Clerici et al., 1995b).

2. Methods

2.1. Cochlear explant harvest

Male and female pigmented guinea pigs, fed ad libitum and housed in an AAALAC accredited vivarium, were

brought into the laboratory and injected with a lethal dose of pentobarbital (50 mg/kg, i.p.). Subjects were decapitated, their bullae rapidly removed from the skull base, and each bulla opened within a vessel of artificial perilymph (AP; 295–305 mOsm; pH 7.2 ± 0.1) containing 10.0 mM D-glucose, 1.5 mM CaCl₂ and 1.5 mM MgCl₂ in Dulbecco's phosphate-buffered saline (Gibco BRL, Grand Island, NY). The AP had been placed overnight in a sealed beaker containing chelating resin (Chelex 100; Sigma), in order to complex iron. The otic capsule was removed under a stereomicroscope, and the modiolar fraction with stria vascularis attached, was transferred to an individual polyethylene microcentrifuge tube, containing 200 µl of AP.

2.2. Ototoxic agents

At this point, 3.0–10.0 µl of AP was removed from the tube, and replaced by an equal volume of ototoxic agent (or control AP solution), appropriate to bring the final concentration of the given ototoxicant to its respective value. Agents under study were: gentamicin sulfate (4.0 mM; dilution of GentocinTM; Shering-Plough, Kenilworth, NJ), kanamycin monosulfate (4.0 mM; Sigma), ethacrynic acid (0.5 mM; Sigma), furosemide (0.3 mM; Sigma), cisplatin (0.1 and 1.0 mM; *cis*-diamminedichloroplatinum; Platinol-AQTM, Bristol-Meyers Squibb), trimethyltin chloride (0.1 mM; ICN Biomedicals, Costa Mesa, CA) and quinine HCl (3.0 mM; Sigma). All chemicals were prepared in AP, except ethacrynic acid, which was first dissolved in chloroform; 3.0 µl of this solution was placed into the 197 µl of AP. Chloroform controls were conducted, as chloroform is known to diminish GSH and GSH-peroxidase activities in isolated hepatocytes (El-Shenawy and Abdel-Rahman, 1993). UV radiation in the EPR experiments was generated by a hand-held UV lamp (Mineralight; multiband UV 254/366 nm; UVP, Inc., San Gabriel, CA), positioned 5" above the microcentrifuge tube.

2.3. Electron paramagnetic resonance spectrometry

The ototoxicant (or UV radiation or control AP solution) exposure, in all cases, lasted for 10 min. Following this incubation, 20 µl of the AP/ototoxicant solution was replaced by an equal volume of the spin trap, 5,5-dimethylpyrroline-*N*-oxide (DMPO; charcoal filtered), which remained in the reaction vessel for 10 min. The AP/DMPO reactive mixture was withdrawn and placed in a quartz flat cell which was then centered in the resonant cavity of a Bruker model 300 spectrometer, having computer-controlled data acquisition and analysis capabilities. The spectrometer was operated at 7.45 mW microwave power, 0.98 G modulation amplitude, 75 G scan width, 14.3 G/s scan rate, 1.28 ms time constant and 9.79 GHz microwave frequency. Magnitude of O₂⁻ or OH[•] formation was char-

acterized in terms of spin adduct peak height (Chan et al., 1994). UV radiation and H_2O_2 (10.0 mM) were utilized as positive control exposure conditions; AP/DMPO alone with explant was the negative control condition. A final condition included AP/DMPO and ototoxicant, but excluded the explanted cochlear tissue. Deferoxamine (1.0 mM; Sigma) was utilized to quench the OH^\cdot signal in parallel samples. Of the 99 EPR trials conducted, at least four were conducted with each agent in the presence of an explant, at least two each also included deferoxamine, and at least two each included the agent, AP/DMPO and no cochlear tissue. Eleven trials were run using explanted material in AP/DMPO alone, and five trials contained only AP/DMPO with no ototoxicant or cochlear explant.

2.4. PeroXOquant™ assay

The PeroXOquant™ Assay (kit #23280; Pierce Chemical Co., Rockford, IL) is a quantitative assay for peroxide formation (Butterfield et al., 1996), whereby hydroperoxides react with sorbitol, converting it to a peroxy radical which catalyzes the oxidation of ferrous (Fe^{2+}) to ferric (Fe^{3+}) iron. Fe^{3+} , in turn, complexes with xylenol orange to yield a purple reaction product; the absorbance is read spectrophotometrically at 560 nm, using a molar absorptivity of $1.5 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$. After the 10 min incubation with or without ototoxicant, cochlear explants were incubated for an additional 30 min with PeroXOquant™ working reagents, in order to quantify peroxide production, as EPR does not identify non-radical species (e.g., peroxides). Graded H_2O_2 standards were utilized as positive controls and to ensure assay linearity. A spectrophotometrically confirmed O_2^- generating system (containing 10 mM Tris-HCl, 8.0 mU xanthine oxidase, 100 μM xanthine and 10 μM EDTA in AP) was also utilized as a positive control exposure solution, to induce H_2O_2 production in cochlear explants (see Clerici et al., 1995a). Explants exposed to AP alone served as negative controls. Following peroxide determination, each explant was homogenized, and its protein content was determined using the method of Lowry et al. (1951) for calculation of peroxide content per mg protein. This protocol has University of Kentucky IACUC approval.

3. Results

The AP/DMPO mixture without the presence of ototoxicant or cochlear explant did not produce spectral peaks indicative of ROS generation. AP/DMPO alone in the presence of cochlear explants (negative controls) also produced no such peaks. Fig. 1 (top trace) illustrates the classic pattern of spectral peaks displayed following OH^\cdot generation — a four line 1:2:2:1 EPR spectrum resulting from equivalent nitrogen and β -hydrogen electron-nuclear hyperfine coupling constants with the DMPO spin adduct

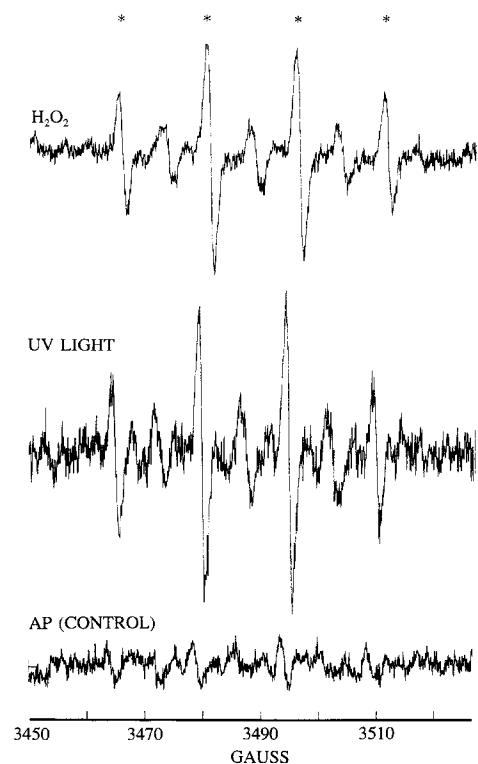


Fig. 1. EPR results illustrate that exposure (10 min) of a cochlear explant in artificial perilymph (AP) containing DMPO to UV radiation or H_2O_2 (10.0 mM), elicited the classic 1:2:2:1 spectral peaks indicative of hydroxyl radical (OH^\cdot) generation (i.e., $\text{OH}^\cdot/\text{DMPO}$ adduct formation). AP/DMPO alone, and cochlea in AP/DMPO without toxicant (bottom trace) conditions did not produce $\text{OH}^\cdot/\text{DMPO}$ adduct spectra. All signals were quenched by deferoxamine (1.0 mM). In this and subsequent figures, asterisks denote the four spectral peaks which uniquely identify the $\text{OH}^\cdot/\text{DMPO}$ adduct. Spectral peak heights are not quantitatively relative; figures only illustrate the presence of $\text{OH}^\cdot/\text{DMPO}$ adduct peaks.

(Butterfield, 1982; Halliwell and Gutteridge, 1985). In this case, H_2O_2 was the initiator for OH^\cdot generation. Background signals (not indicated by asterisks) are derived from the trap itself (Halliwell and Gutteridge, 1985). A second positive control exposure condition, utilizing UV radiation as the exogenous source of ROS initiation, resulted in robust spectra classically indicative of $\text{OH}^\cdot/\text{DMPO}$ adduct formation; this occurred both in the presence and absence of cochlear tissue. This suggests that a minute amount of iron was present, even in the chelexed AP. Fig. 1 also illustrates the low amplitude, apparently random, spectral pattern seen following the AP/DMPO alone plus cochlear explant exposure condition.

Amplification of H_2O_2 – initiated signals occurred in the presence of explants, likely because iron present in cochlear tissues participated in the Fenton chemistry (Fig. 2). Thus, H_2O_2 was able to react with Fe^{2+} to produce Fe^{3+} plus OH^\cdot . Likewise, the 10 min exposure of an explant/AP/DMPO combination to UV radiation produced a large OH^\cdot signal, indicating that UV radiation was able to form OH^\cdot from H_2O_2 . All of these signals were completely quenched in parallel samples by deferoxamine.

Cisplatin and trimethyltin chloride, both metal agents, but which have different structures and reactivities, produced OH[·]/DMPO adduct spectra in the AP/DMPO reactive mixture when cochlear tissue was present (Fig. 2). In the absence of cochlear tissue, trimethyltin chloride exposure did not produce the OH[·]/DMPO adduct signal, however, cisplatin did produce a weak signal when no cochlear material was present. The structure of the cisplatin spectrum, however, also included peaks indicative of non-OH[·]/DMPO adducts; these signals were possibly due to the formation of drug-derived radical species. When these conditions were replicated in the presence of a cochlear explant, the OH[·]/DMPO adduct signal for cisplatin was amplified. In both cases, the inclusion of deferoxamine quenched the OH[·] signals. Fig. 2 illustrates spectral peaks evidenced following cisplatin and trimethyltin chloride exposures.

The two aminoglycoside antibiotics, kanamycin monosulfate and gentamicin sulfate, also produced spectral peaks indicative of OH[·] formation when a cochlear explant was incubated in AP/DMPO along with the given aminoglycoside. Neither kanamycin, nor gentamicin, exhibited OH[·]/DMPO adduct spectral peaks in the AP/DMPO solution without cochlear tissue present. Fig. 3 illustrates spectral peaks seen following the exposure of cochlear explants to kanamycin and gentamicin. The signal for kanamycin was extremely weak relative to background,

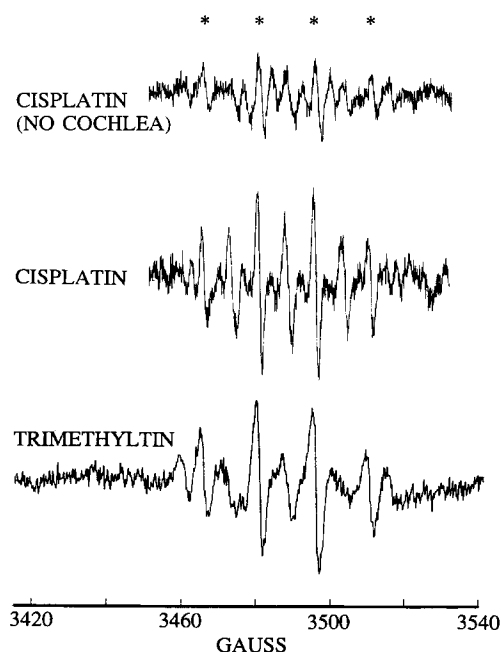


Fig. 2. Illustration of EPR spin trapping spectral peaks indicative of OH[·]/DMPO adduct formation following cisplatin (0.1 mM) in the absence (top trace) and presence (middle trace) of cochlear material. Trimethyltin chloride (0.1 mM) exposures of cochlear explants in the AP/DMPO solution also produced these peaks (bottom trace). The spectrum derived from cisplatin in the presence of cochlear tissue shows a complex structure which may reflect the presence of drug-derived radicals, in addition to OH[·] production.

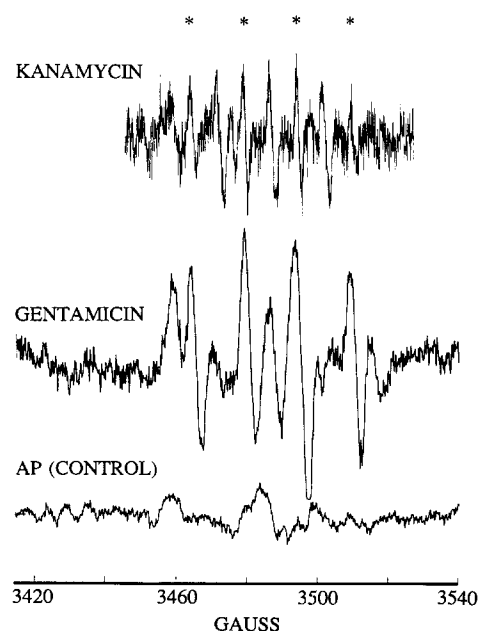


Fig. 3. The aminoglycoside antibiotics, kanamycin monosulfate (4.0 mM) and gentamicin sulfate (4.0 mM), when placed in AP/DMPO with cochlear explants, also initiated the production of OH[·]/DMPO adduct spectral peaks by EPR spin trapping. Kanamycin administrations also evidenced spectral peaks unrelated to OH[·]/DMPO adduct production, although none of the kanamycin-induced signals were very strong relative to background. The bottom trace illustrates the low amplitude, non-specific spectra observed following AP/DMPO treatment of a cochlear explant in the absence of any ototoxicant.

and included other spectra, in addition to the 1:2:2:1 quartet indicative of the DMPO-trapped hydroxyl radical. Kanamycin, thus, produced the least convincing evidence for ROS formation of any agent studied in this investigation. Gentamicin, on the other hand, provided robust signals above the background floor. These signals were reduced to below visual detection by the addition of deferoxamine to the reactive mixture.

Furosemide and ethacrynic acid, two loop diuretics, produced clear OH[·]/DMPO adduct spectral peaks in the presence of cochlear explant tissue (in AP/DMPO; see Fig. 4). Low amplitude peaks were also evident even in the absence of the explant material, but, these signals were amplified in the presence of cochlear tissue, and quenched by the addition of deferoxamine. In the case of ethacrynic acid, controls utilizing chloroform (in AP/DMPO) did not yield an OH[·]/DMPO adduct signal, indicating that the signal seen following ethacrynic acid administration was not due to the presence of the chloroform. Quinine HCl also produced weak OH[·] spectral peaks in the absence of tissue, which were amplified on inclusion of cochlear tissue and diminished when deferoxamine was present (Fig. 4).

The PeroXOquant™ assay results, analyzed using a one-way analysis of variance, demonstrated that the mean amount of peroxide formation at 10 min after the onset of each drug administration to cochlear explants was actually

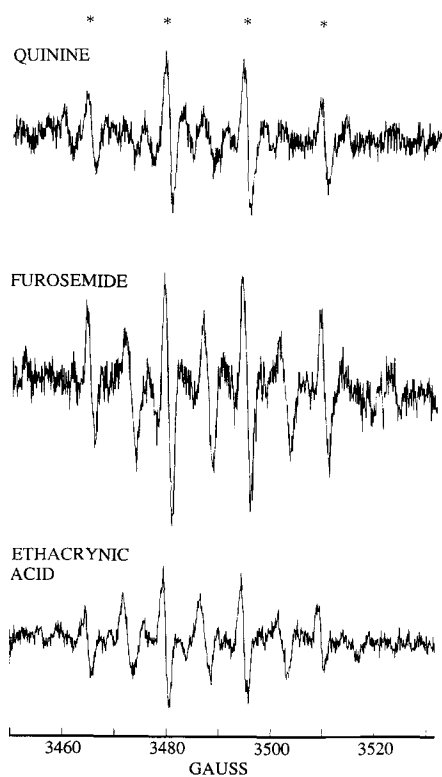


Fig. 4. The EPR spin trapping spectral peaks seen following administration of quinine HCl (3.0 mM) and two loop diuretics, furosemide (0.3 mM) and ethacrynic acid (0.5 mM) to cochlear explants in AP/DMPO produced the OH/DMPO adduct signal when observed by EPR spin trapping.

reduced relative to levels seen after AP control cochlear explant exposures ($F_{6,35} = 2.41$, $P < 0.05$); see Fig. 5. In the cases of gentamicin and kanamycin, peroxide levels

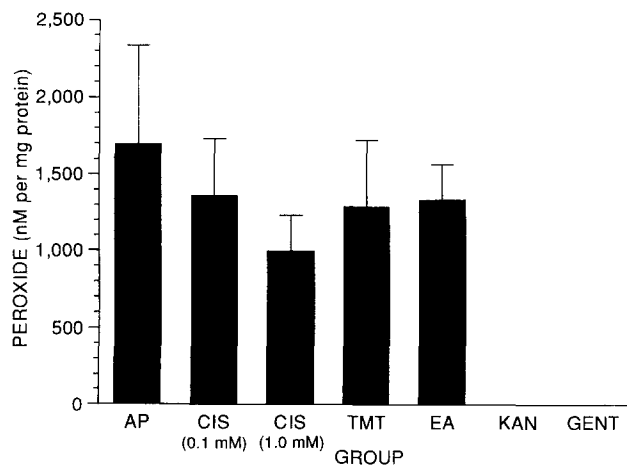


Fig. 5. PeroXOquant™ assay results revealed that mean (\pm SEM) peroxide formation at the end of the 10 min incubation period was lower with each ototoxicant plus an explant, than with AP alone plus an explant. Use of the xanthine–xanthine oxidase system ($n = 7$, data not shown) resulted in a mean peroxide level of $16,311 (\pm 3958)$ nM/mg protein. AP, artificial perilymph ($n = 8$); CIS, cisplatin (0.1 mM, $n = 6$; 1.0 mM, $n = 4$); TMT, trimethyltin chloride ($n = 7$); EA, ethacrynic acid ($n = 7$); KAN, kanamycin ($n = 5$); GENT, gentamicin ($n = 5$).

were reduced to that of the background. In the case of cisplatin, there was a greater reduction in cochlear peroxide content using a 1.0 mM concentration than with a 0.1 mM concentration. Utilization of the xanthine–xanthine oxidase system for O_2^- generation as a positive control led to almost a 10-fold increase in peroxide levels (data not shown). A regression analysis was then performed comparing EPR spectral peak magnitudes and peroxide formation in the cochlear tissue for AP and the various ototoxicants. This analysis revealed only a weak, and not statistically significant ($P > 0.05$), inverse relationship between these indices. It is possible that, at 10 min after the onset of exposure, peroxide is being converted to OH; with the amount of OH \cdot increasing as that of peroxide decreases. However, this question will require further investigation, as the diminished peroxide levels seen following ototoxicant exposures approached the sensitivity limits of the PeroXOquant™ assay.

4. Discussion

Several studies have provided indirect evidence that ROS formation occurs in the cochlea following ototoxicant exposures. The present study is the first to directly detect ototoxicant-induced ROS production in cochlear tissues. These results suggest that agents from several of the classic ototoxicant categories are capable of inducing or promoting metal catalyzed ROS formation in cochlear explants. Free-radical formation, which induces cellular damage in other organ systems, may thus be a mechanism of cochlear cellular damage common to many ototoxicant exposures. Because other studies have demonstrated only partial protection against ototoxicity due to ROS scavenger pretreatments, the formation of oxygen-derived radicals is likely not the only contributor to cochlear damage following such exposures. However, for each of these agents, evidence is accumulating, in cochlear and other tissues, that ROS production is important in their toxicities.

Aminoglycoside exposures produce a predominantly high-frequency cochlear hearing loss, with disruption of the OHC prior to the inner hair cell (IHC), and a base-to-apex progression. Aminoglycosides (or metabolites) enter the perilymph and bind within the OHC (Hiel et al., 1992), where they induce irreversible damage (Schacht, 1986). Such damage may be due to ROS formation, as aminoglycosides have been shown to generate ROS in renal cortical mitochondria, both by increasing H_2O_2 , O_2^- and OH \cdot generation (Walker and Shah, 1987; Du and Yang, 1994), and by enhancing the release of mitochondrial iron, which is then available for metal catalyzed OH \cdot generation (Ueda et al., 1993; Walker and Shah, 1988). Three recent abstracts have extended the iron-mediation hypothesis of ROS production to gentamicin's effects in the cochlea. The first showed that gentamicin and kanamycin enhance production of OH \cdot in cochlear explants, which strongly sug-

gests a role for iron catalyzed redox cycling in their ototoxicities (Clerici et al., 1995b). The other two abstracts showed that gentamicin is a weak iron chelator (Wang et al., 1996), and that the administration of other iron chelators and ROS scavengers protect against gentamicin-induced ototoxicity (Song and Schacht, 1996); these abstracts suggest that a gentamicin-iron complex may be the redox active source of ROS formation in gentamicin-induced ototoxicity. ROS scavengers protect against gentamicin-induced nephrotoxicity (Nakajima et al., 1994), and both kanamycin-induced ototoxicity (Pierson and Møller, 1981) and *in vitro* OHC disruption (Mizukoshi and Asano, 1976). In addition, GSH administration protects against cochlear damage and dysfunction following to both *in vivo* (Garetz et al., 1994a) and *in vitro* (Garetz et al., 1994b) gentamicin exposures, as do ROS scavenger administrations *in vitro* (Dulon et al., 1989). However, although ROS generation is known to damage the OHC plasma membrane (Clerici et al., 1995a), effects upon the IHC, type I spiral ganglion cell (SGC₁) and stria vascularis have not been studied. Data from our laboratory (Clerici and Yang, 1996a,b) indicate that a primary acute effect of cochlear ROS generation is to reduce compound action potential threshold sensitivity, which suggests that damage occurs to the IHC/SGC₁ unit.

Cisplatin also preferentially destroys OHCs in the basal cochlea, where it binds to the OHC (Komune et al., 1981), and within the stria vascularis (Schweitzer, 1993). There is evidence that cisplatin kills tumor cells by binding to DNA and producing ROS, which cleave DNA strands in the aerobic fraction of the tumor (Brandon and Dabrowiak, 1984), in a manner similar to X-irradiation. Cisplatin ototoxicity is potentiated by concomitant X-ray therapy (Baranak et al., 1988), possibly because of synergistic ROS production. Cisplatin promotes ROS generation in macrophages (Oyanagui, 1977), and subsequent malondialdehyde formation in brain, liver and small intestines after *in vivo* administration (Torii et al., 1993) and in renal cortical slices (Hannemann and Baumann, 1988) *in vitro*. Cisplatin also increases lipid peroxidation and depletes GSH in renal mitochondria (Zhang and Lindup, 1993). In the cochlea, cisplatin exposure increases levels of superoxide dismutase, catalase and malondialdehyde, and decreases those of GSH, GSH-peroxidase and GSH-reductase (Ravi et al., 1995), thus, implicating cochlear ROS formation in cisplatin-induced ototoxicity. This is supported by the evidence that ROS scavengers protect against cisplatin-induced hearing dysfunction, OHC loss (Otto et al., 1988; Schweitzer, 1993), and nephrotoxicity (Yuhas and Culo, 1980). The greater aerobic:glycolytic metabolism in the basal cochlea (Thalman et al., 1973), might render this portion of the organ more susceptible to ROS formation and damage, and play a role in the preferential high-frequency hearing losses induced by the aminoglycosides and cisplatin.

Trimethyltin is an environmental ototoxicant which

damages OHCs of the basal cochlea, but in addition, trimethyltin attacks the myelin sheathes of the SGC₁ (Fechter et al., 1992). SGC₁ damage might be responsible for the broad mid-to-high-frequency auditory disruption seen following trimethyltin administration (Clerici et al., 1991; Clerici, 1996), as the free fatty acids contained in both the myelin and the plasma membrane are potential targets of ROS-induced lipid peroxidation. Trimethyltin induces ROS generation in crude synaptosomal preparations (LeBel and Bondy, 1991; Ali et al., 1992), and its neurotoxicity is modulated by altering dietary levels of ascorbic acid, an important antioxidant (Bannon et al., 1993); organotins also alter membrane lipids and proteins (Butterfield et al., 1991). In various cell types, trimethyltin causes bleb formation and arachidonic acid formation (Kafer et al., 1992), consistent with ROS formation. In the cochlea, trimethyltin induces OHC bleb formation *in vivo* (Fechter et al., 1992) and *in vitro* (Clerici et al., 1993) as does *in vitro* administration of specific ROS generating systems (Clerici et al., 1995a). These data, in addition to that which shows that superoxide dismutase pre-administration partially protects against the acute, high-frequency auditory disruption induced by trimethyltin (Clerici, 1996), suggest that TMT-induced cochlear damage is partially ROS-mediated.

Loop diuretics produce a temporary hearing loss and primarily disrupt stria vascularis function (Bosher, 1980), by blocking Na⁺,K⁺,2Cl⁻ cotransport. However, ethacrynic acid is also utilized experimentally to deplete mitochondrial and cytosolic GSH (Redegeld et al., 1992). These compounds react with thiol groups deeply embedded in the plasma membrane, a feature believed to underlie their ability to both disrupt OHC electromotility *in vitro* (Kalinec and Kachar, 1993), and induce bleb formation in OHCs (Yang and Clerici, 1996) and other cell types; the latter is believed to depend upon thiol oxidation (Malorni et al., 1991). Similar surface bleb formation occurs in isolated OHCs following exposure to ROS generating systems (Clerici et al., 1995a). That cellular shape changes and underlying cytoskeleton disruption due to ethacrynic acid exposure are prevented by concomitant exposure to cysteine (Erickson-Lamy et al., 1992), suggests that such damage is ROS-related, possibly due to thiol oxidation.

Ethacrynic acid disrupts mitochondrial distribution and morphology in many cell types (Soltys and Gupta, 1994), and induces lipid peroxidation, for which deferoxamine provides protection (Dhanbhoora and Babson, 1992). Increased lipid peroxidation is believed due, not to oxidant properties of ethacrynic acid, but to GSH depletion, which permits mitochondrial H₂O₂ to participate in iron-mediated OH[·] production (Dhanbhoora and Babson, 1992). Furosemide also generates reactive intermediates in microsomes, which may underlie both its binding and toxic properties in the kidney (Wirth et al., 1975). Loop diuretics potentiate aminoglycoside ototoxicity (Brummett et al., 1975), where synergistic ROS formation may play a role,

as disruption of the GSH reducing system potentiates the hearing loss induced by the combination of kanamycin and ethacrynic acid (Hoffman et al., 1988). That the hearing loss induced by furosemide and ethacrynic acid is usually reversible, may be because these agents target the stria vascularis, rather than the hair cells or SGC₁s.

Quinine, and its derivatives such as chloroquine and primaquine, kill only pigment forming malarial parasites, and like the loop diuretics, produce a temporary hearing loss and bind in the stria vascularis (Denker and Lindquist, 1975), specifically to melanin. Melanin is a unique, pigmented biopolymer, being a stable free radical, capable of binding other transient free radicals (Commoner et al., 1954). Buszman et al. (1984) showed, using EPR spectrometry, that the amount of chloroquine binding to melanin is directly proportional to the free-radical content of the uncomplexed melanin. Quinine's chemical structure is similar to that of the quinones, which undergo oxidation-reduction reactions in the mitochondrial electron transport chain, suggesting that quinine and its derivatives might have an extremely high redox potential. Primaquine and its metabolites induce the formation of H₂O₂, quinone-amine derivatives, drug-derived radicals, and OH· via metal ion catalyzed redox cycling (Vasquez-Vivar and Augusto, 1992; Grinberg and Samuni, 1994). Both quinine and primaquine also decrease oxygen consumption, increase lactate dehydrogenase leakage and alter ion exchange in renal cortical slices (Mulchinski et al., 1994), which suggests that these agents are capable of initiating ROS formation.

In summary, several lines of circumstantial evidence implicate ROS formation and oxidative damage in ototoxicity. ROS scavengers and GSH inhibitors modulate the cochlear dysfunction and structural damage seen following exposures to many of these agents, as they do also to cochlear ischemia-reperfusion (Seidman et al., 1991; Seidman and Quirk, 1991) and noise exposures (Seidman et al., 1993). Many ototoxic agents also produce damage via ROS formation in other organs. In vitro studies demonstrate that similar forms of damage seen in isolated OHCs also occur following exposures both to these ototoxicants and to other conditions which might engender ROS formation. In addition, high cochlear levels of antioxidant enzymes (Pierson and Gray, 1982), and the ongoing synthesis of melanin, suggest that the inner ear contains a detoxification system capable of protecting against ROS-related damage.

However, the present study is the first direct demonstration that ototoxicants from a variety of categories can promote the generation of ROS in cochlear tissue and, in particular, the metal catalyzed formation of OH·. Each of these agents likely has damaging effects beyond, although possibly related to, ROS formation (potentially including altered Ca²⁺ flux and excitatory amino acid accumulation), so that, the specific contribution of ROS toward cochlear damage remains to be determined. Yet, the detection of

enhanced ROS formation following a variety of xenobiotic exposures permits a better understanding of how a common mechanism of damage might contribute to cochlear disruption.

Acknowledgements

The authors thank Dr. Lihua Yang for technical contributions. This research was funded by the Division of Otolaryngology-Head and Neck Surgery, Department of Surgery, University of Kentucky College of Medicine and by Grant DC06701 from the National Institute of Environmental Health Sciences (NIEHS), NIH. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIEHS, NIH.

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