BIOMEDICAL AND ENVIRONMENTAL SCIENCES **18**, 198-206 (2005)

Partial Protection by Lipoic Acid Against Carboplantin-induced Ototoxicity in Rats[1]

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Objective To investigate the alterations in auditory brainstem evoked responses (ABRs) and the changes of carboplatin-induced ototoxicity in the cochlear oxidant/antioxidant systems and otoprotection by an antioxidant lipoate. **Methods** Male wistar rats were divided into four groups and treated as follows: 1) vehicle (saline) control, 2) carboplatin (256 mg/kg, i.p.), 3) lipoate (100 mg/kg, i.p.), 4) lipoate + carboplatin. Post-treatment ABRs were performed after four days and rats were sacrificed with their cochleae harvested and analyzed. **Results** Carboplatin significantly elevated ABR threshold above the pretreatment thresholds. Lipoate+carboplatin treated rats showed decreased elevation of hearing threshold. Carboplatin significantly depleted cochlear reduced to oxizized glutathione (GSH/GSSG) ratio, whereas lipoate+carboplatin treatment increased GSH/GSSG ratio. Carboplatin significantly decreased cochlear copper zinc-superoxide dismutase (CuZn-SOD), catalase (CAT), glutathione peroxidase (GSH-Px), glutathione reductase (GR) and glutathione-S-transferase (GST) activities and enzyme protein expressions and a significant increase in Mn-SOD activity, protein expression and malondialdehyde (MDA) level. Cochlear antioxidant enzyme activities, enzyme protein expressions and MDA level were partially restored in lipoate+carboplatin treated rats, compared to carboplatin alone. **Conclusion** Carboplatin-induced ototoxicity is related to impairment of cochlear antioxidant system and otoprotection conferred by lipoate is associated with partial sparing of the cochlear antioxidant defense system.

Key words: Carboplatin; Alpha-lipoic acid; Ototoxicity; Antioxidants; Rats

INTRODUCTION

Carboplatin [cis-diamine (1,1-cyclobutanedicarboxylate) platinum (II)] is currently being used in clinic as an alternative anti-cancer drug for the treatment of a variety of cancers such as small-cell lung cancer, ovarian cancer, carcinomas of head and neck as well as other types of cancers[1-3]. The identification of dose escalation of carboplatin is an important factor in achieving optimal anti-neoplastic effects[4-5]. Single or repeated chemotherapy at high doses of carboplatin result in ototoxicity as a toxic side effect in cancer patients[6-8]. Carboplatin-induced ototoxicity has also been demonstrated in experimental animals such as rats, guinea pigs and chinchillas[9,12]. We have recently reported that carboplatin-induced dose-dependent ototoxicity is related to oxidative injury to the cochlea in a rat model[13,19].

The use of chemoprotectors has been evaluated to alleviate the severity of the toxic side effects of carboplatin such as haematological and gastrointestinal toxicity, nephrotoxicity and neurotoxicity. Most of the chemoprotectors that have been evaluated in experimental and/or clinical studies include sodium thiosulphate, amifostine, glutathione, norepinephrine, diethyldithiocarbamate and immunomodulator AS101[4,8,13-16]. Although most of these sulfur-containing compounds provide protection against carboplatin toxicity by reacting with platinum metal, these compounds also exert their own side effects in humans and experimental animals.

Alpha-lipoic acid, an essential cofactor for mitochondrial enzymes has been proven as a novel biological antioxidant and a potent free radical scavenger[17-18]. Exogenous administration of this agent has been found to have therapeutic potential in neurodegenerative disorders, heavy metal toxicity and oxidative tissue injury[18-19]. Alpha-lipoic acid has been shown to protect against cisplatin-induced ototoxicity in rats[20]. This study hypothesizes that

[1]This work was supported in part by the Central Research Committee (CRC) Grant of SIU School of Medicine.

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0895-3988/2005
CN 11-2816/Q

carboplatin-induced ototoxicity is associated with the depletion of cochlear antioxidant defense system, enhanced lipid peroxidation and elevated ABR thresholds in rats. The partial protection conferred by a biological antioxidant alpha-lipoic acid is associated with preservation of cochlear antioxidant defense system and ABR thresholds. Therefore, this study was designed (i) to determine the changes in the concentrations of endogenous antioxidant (GSH), antioxidant enzyme activities and enzyme protein expressions (CuZn-SOD, Mn-SOD, CAT, GSH-Px, GR, and GST) and the concentrations of the end product of lipid peroxidation (MDA) in the cochleae to changes in ABRs in rats treated with carboplatin and (ii) to evaluate the oto-protective efficacy of a biological antioxidant alpha-lipoic acid against carboplatin-induced biochemical changes in the cochlea of rats.

METHODS

Chemicals

Chemicals such as GSH, oxidized glutathione (GSSG), and gamma-glutamyl glutamate; 1,1,1,1-tetraethoxy-propane, enzymes (CuZn-SOD, Mn-SOD; CAT, GSH-Px, and GR), solvents of analytical grade (methanol and glacial acetic acid) used for HPLC, carboplatin, alpha-lipoic acid, monoclonal antibody for CuZn-SOD, GST, peroxidase-conjugated secondary antibody were purchased from Sigma Chemicals (St. Louis, MO). Monoclonal antibodies for Mn-SOD, GSH-Px and CAT were purchased from Biodesign Int., Kennebunk, ME and Oxis Health Products Inc., Portland, OR, respectively. Coomassie protein assay reagent was purchased from Pierce Company (Rockford, IL).

Animals

Male wistar rats (250-300 g) were obtained from Charles River (Wilmington, MA) and divided into four groups and treated as follows: 1) control (vehicle saline, pH 7.6, 1 mL/kg) single intraperitoneal (i.p.) bolus administration (n=7); 2) carboplatin at a dose of (256 mg/kg and volume of 1 mL/kg, i.p.) bolus administration (n=7); 3) alpha-lipoic acid (dissolved in alkaline saline) at a dose of (100 mg/kg, and volume of 1 mL/kg, i.p.) was injected 30 min before saline administration (n=5); and 4) alpha-lipoic acid (100 mg/kg, i.p.) was injected 30 minutes before carboplatin administration (n=7). Pretreatment ABRs were performed in rats from all groups while they were under xylazine: ketamine sedation, which were followed by the various drug treatments described above. Post-treatment ABRs were performed four days later (maximum ototoxicity observed on the 4th day, unpublished observations) and the data were compared to the pretreatment ABRs for changes in thresholds. Thus, each animal served as its own control for the ABRs. The rats in all the groups were sacrificed four days post-treatment and the cochleae harvested and frozen in liquid nitrogen and stored at -80℃ until analysis could be completed.

Auditory Brain Stem Evoked Responses (ABRs)

Rats were sedated with a Rompun Cocktail (xylazine, ketamine: 3.4 mg/kg, 172.4 mg/kg). Control ABRs were measured using a DEC PDP 11/73 (Digital Equipment Corporation, NH) based signal generating/averaging system in response to 100 sec clicks and tone pips at 2, 4, 8, 16, and 32 kHz, which were of 10 ms plateau with a 1 ms rise fall time. The stimuli were presented inside a double wall radio frequency shielded sound booth using an Etymotic ER-2 earphone placed directly into the ear canal. Clicks and tone pips were presented at a rate of 5× /sec. Stimulus intensities were measured using a Bruel and Kjaer sound pressure level meter (model 2209) with a 1/4 microphone (model 4136) inside an artificial ear canal (RE: 20 micro Pascals). Intensities were expressed in decibels (dB) sound pressure level (SPL) peak equivalent, based on the calibration. Animals were presented with a stimulus intensity series, which was initiated at 10 dB SPL and reached a maximum of 90 dB SPL. Stimulus intensity was progressively increased in 10 dB increments and the resulting ABRs were observed on a video monitor. Intensities that appeared to be near threshold were repeated. Threshold was defined as the lowest intensity capable of producing a visually detectable, reproducible response. Threshold responses typically displayed wave IV and/or a wave II/III complex. There was some variation due to electrode placement and stimulus frequency. The voltage associated with threshold was 0.5 V. Sub-dermal electrodes were used to record brain potentials differentially. The active lead was positioned at the vertex and referred to the second electrode at the tip of the nose. The ground electrode was located over the neck muscles. Potentials were amplified 1 000 times inside the sound attenuation booth (bandwidth, 0.1 Hz to 10 kHz) and signals were further amplified to produce an overall gain of approximately 100 000 and viewed on an oscilloscope. Care was taken to ensure that the band pass of the entire system included those frequencies that represent the ABR. The ABRs were sampled for 20.5 ms following stimulus onset. Stimuli were repeated 5×/sec and a total of 512 trials were averaged using an analog to digital converting system. Evoked potentials were recorded before drug

administration and three days post administration. The ABR measurement in control rats proved to be highly reproducible in the retest schedule, indicating high inter-test reliability[21-22].

Determination of GSH and Its Disulfide (GSSG) by High Pressure Liquid Chromatography (HPLC)

The concentrations of GSH and GSSG were determined in the tissues by a modified HPLC method of Fariss and Reed[23]. Two hundred and fifty L of the tissue-acid extract containing internal standard (gamma-glutamyl glutamate) was mixed with 100 L of 100 mmol/L iodoacetic acid in a 0.2 mmol/L m-cresol purple solution. This acidic solution was brought to basic conditions (pH 8.9) by the addition of approximately 400 L of 2 mol/L KOH - 2.4 mol/L $KHCO_3$. The sample was placed in the dark at room temperature for 1 hour. Rapid S-carboxymethyl derivatization of GSH, GSSG and gamma-glutamyl glutamate occurred soon after the change in pH. N-dinitrophenyl derivatization of the samples was obtained by incubation for 12 hours at 4℃ in the presence of 1% 1-fluoro-di-nitrobenzene. Multiple samples were analyzed using the ISCO auto sampler controlled by ISCO Chemical research program. The sensitivity of the HPLC for GSH was 50 picomol/injection volume and 25 picomol/ injection volume for GSSG.

Enzyme Assays

SOD activity was determined at room temperature according to the method of Misra and Fridovich[24]. Ten L of tissue extract was added to 970 l (0.05 mol/L, pH 1: 0.2, 0.1 mmol/L EDTA) carbonate buffer. Twenty L of 30 mmol/L epinephrine (dissolved in 0.05% acetic acid) was added to the mixture and SOD was measured at 480 nm for 4 min on a Hitachi U-2000 spectrophotometer. The rate of the reaction was calculated where linearity occurred, usually between 90 and 180 seconds. SOD activity was expressed as the amount of enzyme that inhibits the oxidation of epinephrine by 50%, which is equal to 1 unit. Mn-SOD activity was determined by adding 100 L of 20 mmol/L NaCN to inhibit CuZn-SOD activity. CuZn-SOD activity was determined by subtracting the Mn-SOD from total SOD activity.

CAT activity was determined at room temperature by a slight modification of a method of Aebi[25]. Ten L ethanol was added per 100 L of tissue extract (dissolved in 0.5 mol/L, pH 7.0, 0.1 mmol/L EDTA, phosphate buffer), and then placed in an ice bath for 30 min. Then 10 L of Triton X 100 RS was added per 100 L of the tissue extract. Ten L of tissue extract was added in a cuvette containing 240 L phosphate buffer and 250 L (0.066 mol/L) H_2O_2 (dissolved in phosphate buffer) and measured at 240 nm for 30 seconds. The molar extinction coefficient of 43.6 mmol/L · cm^{-1} was used to determine CAT activity. One unit of CAT activity was defined as the m moles of H_2O_2 degraded/min/mg protein.

GSH-Px activity was determined by a method of Flohe and Gunzler[26] at 37℃. All reaction mixtures were dissolved in 0.05 mol/L, pH 7.0, 0.1 mmol/L EDTA phosphate buffer. A reaction mixture consisted of 500 L phosphate buffer, 100 L 0.01 mol/L of glutathione (GSH), 100 L 1.5 mmol/L NADPH, and 100 L glutathione reductase (0.24 units). One hundred L of the tissue extract was added to the reaction mixture and incubated at 37℃ for 10 minutes. Then 50 L of 12 mmol/L t-butyl hydroperoxide was added to the tissue reaction mixture and measured at 340 nm for 180 seconds. The millimolar extinction coefficient of 6.22 mmol/L · cm^{-1} was used to determine the activity of GSH-Px. One unit of activity was equal to the millimoles of NADPH oxidized/min/mg protein.

GR activity was determined by the method of Carlberg and Mannervick[27] at 37℃. Fifty L of NADPH (2 mmol/L) in 10 mmol/L Tris-HCl buffer (pH 7.0) added in a cuvette containing 50 L of GSSG (20 mmol/L) in phosphate buffer (0.5 mol/L, pH 7.0, 0.1 mmol/L EDTA), and 800 L of phosphate buffer were incubated at 37℃ for 10 min. One-hundred L of tissue extract was added to the NADPH-GSSG buffered solution and measured at 340 nm for 3 minutes. The millimolar extinction coefficient of 6.22 cm^{-1} was used to determine the activity of GR. One unit of GR activity was equal to the millimoles of NADPH oxidized/min/mg protein.

GST activity was assayed by the method of Habig *et al.*[28] using 10 mmol/L 1-chloro-2, 4-dinitro benzene (CDNB) as substrate. Fifty L of tissue homogenate was added to 750 L 0.1 mol/L phosphate buffer containing 0.1 mmol/L EDTA and 100 L of 10 mmol/L GSH. One hundred L of CDNB was added to start the reaction. The changes in optical density were recorded at 340 nm for 3 min. The enzyme activity was calculated using extinction coefficient 9.6 mmol/L · cm^{-1} and expressed as u moles of CDNB utilized/min/mg protein.

Antioxidant Enzyme Protein Levels By ELISA

The antioxidant enzyme (CuZn-SOD, Mn-SOD, CAT, GST and GSH-Px) protein levels were determined using enzyme linked immunosorbent assay (ELISA) technique[12]. Tissue extracts (0.05 mL) prepared in phosphate buffered saline (PBS) (10 mmol/L phosphate buffer, pH 7.4, 150 mmol/L NaCl and 0.1% sodium azide) were pipetted into each well of polyvinyl microtiter plate and incubated overnight

at 4°C. Coating solution was removed and washed 3 times with washing buffer (10 mmol/L phosphate buffer, pH 7.4, 150 mmol/L NaCl, 0.05% Tween 20) and distilled water. One-hundred μL of monoclonal antibody (CuZn-SOD) (Sigma Chem. Co., MO) diluted in PBS (1:300) or other diluted (1:300) antibodies viz. anti-Mn-SOD, anti-catalase, anti-glutathione peroxidase, and anti-GST respectively, were added to each well, incubated at room temperature for 2 h, and washed three times as before. One-hundred μL of peroxidase conjugated secondary antibody diluted in PBS (1:100) was added to each well, incubated for 2 h and washed three times as before. One-hundred μL of substrate (1% H_2O_2 and 1 mg/mL 5-amino salicylic acid) in reaction buffer (0.02 mol/L phosphate buffer, pH 6.8) was added to each well and incubated for 30 min. The reaction was stopped by adding 0.1 mL of 3 nmol/L NaOH and absorption of the microtiter wells was read at 450 nm using an ELISA reader (Automated Microplate Reader, Model EL311, Bio-Tek Instruments, Inc., Winooski, VT).

Lipid Peroxidation Assay

The end product of lipid peroxidation [malondialdehyde (MDA)] was estimated by the method of Ohkawa *et al.*[29]. One hundred μL of tissue homogenate was added to 50 L of 8.1% sodium dodecyl sulfate, vortexed and incubated for 10 min at room temperature. Three hundred seventy five L of 20% acetic acid and 375 L of thiobarbituric acid (0.6%) were added and placed in boiling water bath in sealed tubes for 60 min. The samples were allowed to cool at room temperature. 1.25 mL of n-butanol: pyridine (15:1) was added, vortexed and centrifuged at 1 000 rpm for 5 min. Five hundred L of the colored pink layer was measured at 532 nm on spectrophotometer using 1,1,3,3-tetra-ethoxypropane as standard. Malondialdehyde (MDA) concentration was expressed as n moles/mg protein.

Protein Assay

Protein concentration was estimated according to the method of Read and Northcole[30] using bovine serum albumin as a standard.

Statistical Analysis

The data were expressed as $\bar{x} \pm s$. The data for biochemical parameters were analyzed statistically using two-way analysis of variance (ANOVA) followed by Duncan's multiple range test using the SAS statistical software package (SAS Institute, Cary, NC) for comparison of treated groups with control groups (saline and alpha-lipoic acid). The data of ABR were subjected to statistical analysis using two-tailed *t*-test. The 0.05 level of probability was used as the criterion for statistical significance.

RESULTS

The changes in ABR thresholds in control, carboplatin, alpha-lipoic acid and alpha-lipoic acid plus carboplatin treated rats are depicted in Fig. 1. Carboplatin significantly (P<0.05-0.001) elevated ABR thresholds for clicks, 2 kHz, 4 kHz, 8 kHz, 16 kHz and 32 kHz tone burst which were 12.22±2.1 dB, 6.66±2.9 dB, 8.88±4.5 dB, 10.00±3.5 dB, 11.11±2.0 dB and 15.00±3.5 dB, respectively. A partial but significant reduction of ABR threshold was observed in the carboplatin plus alpha-lipoic acid treated groups (P<0.05). The ABR threshold changes in animals treated with alpha-lipoic acid (100 mg/kg) plus saline for clicks, 2 kHz, 4 kHz, 8 kHz, 16 kHz and 32 kHz tone bursts were 0.20±0.05 dB, 0.30±0.04 dB, 0.10±0.02 dB, 0.20±0.05 dB, 0.10±0.05 dB, and 0.20±0.05 dB, respectively. For saline control group the ABR threshold changes were 0.25±0.05 dB, 0.35±0.04 dB, 0.15±0.05 dB, 0.25±0.05 dB, 0.15±0.05 dB, and 0.20±0.05 dB, respectively. The changes in thresholds of ABRs suggested partial protection by alpha-lipoic acid specifically at higher frequencies (16 kHz and 32 kHz) against carboplatin-induced ototoxicity in rats.

The changes in GSH/GSSG ratio in control, carboplatin, alpha-lipoic acid and alpha-lipoic acid plus carboplatin treated rats are depicted in Fig. 2. Cochlear GSH/GSSG ratio significantly (P<0.05) decreased after carboplatin treatment compared to control (Fig. 2). The cochlear GSH/GSSG ratio was significantly increased in lipoate plus carboplatin treated group compared to carboplatin alone group (P<0.001). In rats treated with only alpha-lipoic acid plus saline, GSH/GSSG ratio was just above the control value.

The changes in cochlear MDA levels in carboplatin, alpha-lipoic acid and alpha-lipoic acid plus carboplatin treated rats are depicted in Fig. 3. Cochlear MDA concentrations significantly increased in the carboplatin group compared to control group (P<0.001). The cochleae of rats administered alpha-lipoic acid plus carboplatin had lower MDA concentrations than those of rats injected with carboplatin alone. The cochlear MDA concentration in rats treated with only alpha-lipoic acid plus saline was close to the control value.

The changes in cochlear antioxidant enzyme activities in carboplatin, alpha-lipoic acid and alpha-lipoic acid plus carboplatin treated rats are depicted in Table 1. Cochlear CuZn-SOD activity significantly decreased after carboplatin treatment (P<0.05). The cochlear CuZn-SOD activity in carboplatin plus alpha-lipoic acid group showed a

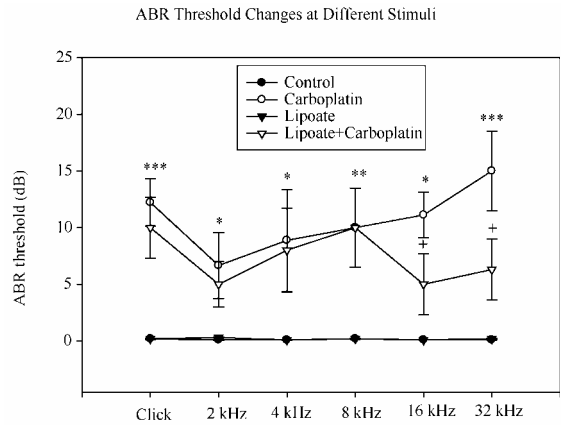

FIG. 1. ABR threshold (dB) changes at click, 2 kHz, 4 kHz, 8 kHz, 16 kHz, and 32 kHz stimuli. ABR thresholds were measured in all pretreated rats. Post treatment thresholds were measured four days after carboplatin administration [-○-] (n=7), or four days after lipoate (100 mg/kg) plus carboplatin treatment [-▽-] (n=7), lipoate (100 mg/kg) plus saline treatment [-▼-] (n=5) and saline control [-●-] (n=7). (*) = significantly different from control or lipoate group (P<0.05), (**) = significantly different from control or lipoate group (P<0.01), (***) = significantly different from control or lipoate group (P<0.001), (+) = significantly different from carboplatin group (P< 0.05).

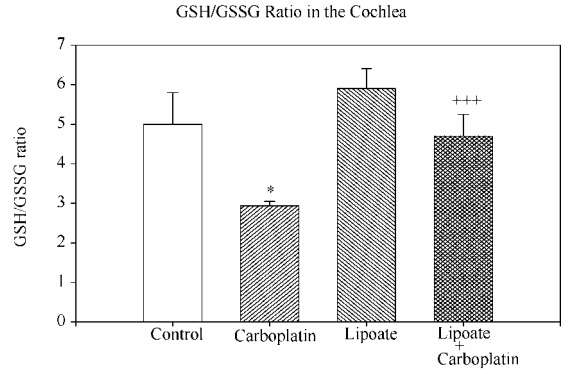

FIG. 2. Changes in cochlear reduced to oxidized glutathione (GSH/GSSG) ratio in control, carboplatin, and carboplatin plus lipoate (100 mg/kg) injected rats. The samples were taken four days post treatment. Cochlear glutathione significantly decreased in carboplatin was treated rats (n=7, $^{*}P$<0.05) as compared to control. Cochlear GSH/GSSG ratio in rats administered lipoate (100 mg/kg) plus carboplatin significantly different from carboplatin treatment (n=7, +++P<0.001). Values were expressed as $\bar{x} \pm s$.

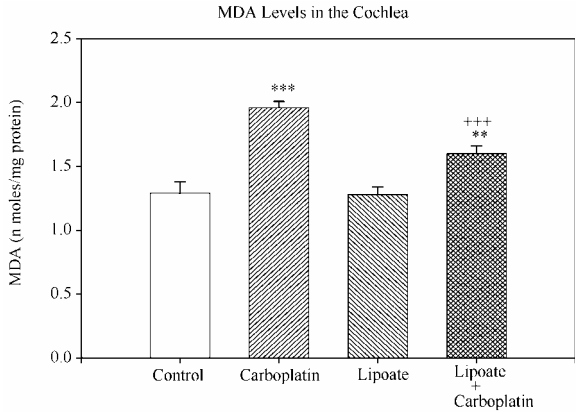

FIG. 3. Changes in cochlear MDA concentrations in control, carboplatin, and lipoate (100 mg/kg) plus carboplatin injected rats. The samples were taken four days post treatment. Cochlear MDA concentrations increased significantly (n=7, ***P<0.001) in carboplatin treated rats as compared to control or lipoate group. In the lipoate (100 mg/kg) plus carboplatin injected rats, cochlear MDA concentration significantly decreased (n=7, **P<0.02) compared to control or lipoate group and (n=7, +++P<0.001) compared to carboplatin group. Values were expressed as $\bar{x} \pm s$.

significant increase compared to carboplatin group (P <0.05). The cochlear CuZn-SOD activity was close to control in alpha-lipoic acid plus saline treated rats. Cochlear Mn-SOD activity significantly increased after carboplatin treatment compared to control (P<0.05). Cochlear Mn-SOD activity significantly increased in lipoate plus carboplatin group compared to the control group (P<0.05). Cochlear CAT and GSH-Px activities significantly decreased in carboplatin injected rats compared to control group (P<0.02) and (P<0.01), respectively. Cochlear CAT and GSH-Px activities in alpha-lipoic acid plus carboplatin group significantly increased compared to control group (P<0.05) and carboplatin group (P<0.01), respectively. Cochlear GR and GST activities significantly decreased after carboplatin treatment (P<0.02). The cochlear GR and GST activities of rats injected with carboplatin plus alpha-lipoic acid had a significantly higher activity than cochleae of rats injected with carboplatin alone (P<0.05). The changes in antioxidant enzyme protein expressions in control, carboplatin, alpha-lipoic acid and alpha-lipoic acid plus carboplatin treated rats are depicted in Table 2. Cochlear CuZn-SOD protein level significantly decreased after carboplatin treatment (P<0.001) as compared to control. The CuZn-SOD protein level in the cochleae of rats administered alpha-lipoic acid plus carboplatin showed a significant increase compared to the carboplatin group (P<0.001). The cochlear CuZn-SOD protein level was close to control in alpha-lipoic acid plus saline treated rats. Cochlear Mn-SOD protein level significantly increased after carboplatin treatment compared to control (P<0.02). Cochlear Mn-SOD protein level significantly increased in lipoate plus carboplatin group compared to the carboplatin alone group (P<0.02). The cochlear Mn-SOD protein level was just above to controls in alpha-lipoic acid plus saline treated rats. Cochlear CAT and GSH-Px protein levels significantly decreased in carboplatin injected rats (P<0.02) compared to control group. Cochlear CAT and GSH-Px protein levels in alpha-lipoic acid plus carboplatin significantly increased compared to carboplatin alone (P<0.05). Cochlear GST protein level significantly decreased after carboplatin treatment (P<0.05) compared to control group.

TABLE 1

Effects of Carboplatin, Lipoate, and Lipoate Plus Carboplatin on Antioxidant Enzyme Activities in the Cochlea of Rats Four Days Post Treatment ($\bar{x} \pm s$)

Enzyme Activities	Control (Saline)	Carboplatin	Lipoate	Lipoate+Carboplatin
Copper Zinc-SO	19.0±3.5	10.7±2.6[a]	18.4±1.4	16.9±1.8[d]
Manganese-SOD	10.6±0.8	13.9±1.0[a]	11.5±1.2	12.4±0.9[a]
Catalase	33.7±3.6	18.7±3.9[b]	32.7±3.0	24.3±2.8[a]
Glutathione				
Peroxidase	56.0±10.7	21.7±3.0[c]	54.5±5.9	45.0±6.2[e]
Glutathione	15.4±1.6	8.1±1.8[b]	16.7±1.8	12.5±1.2[d]
Reductase				
Glutathione-S-transferase	16.3±0.7	8.7±2.4[b]	15.4±0.9	13.8±0.8[a,d]

Note. Enzyme activities were expressed as units/mg protein. [a]P<0.05 compared to control or lipoate group; [b]P<0.02 compared to control or lipoate group; [c]P<0.01 compared to control or lipoate group; [d]P<0.05 compared to carboplatin group; [e]P<0.01 compared to carboplatin group.

TABLE 2

Effects of Carboplatin, Lipoate, and Lipoate Plus Carboplatin on Antioxidant Enzyme Protein Levels (g/mg protein) in the Cochlea of Rats Four Days Post-treatment ($\bar{x} \pm s$)

Enzyme Proteins	Control (Saline)	Carboplatin	Lipoate	Lipoate+Carboplatin
Copper Zinc-SOD	1.5±0.1	0.6±0.09[c]	1.6±0.1	1.1±0.09[a,f]
Manganese-SOD	0.5±0.08	0.9±0.02[b]	0.6±0.08	0.7±0.04[a,e]
Catalase	4.5±0.5	2.6±0.4[b]	4.7±0.5	4.0±0.3[d]
Glutathione Peroxidase	6.6±0.5	4.1±0.7[b]	6.5±0.5	5.9±0.4[d]
Glutathione-S- transferase	4.4±0.4	3.3±0.2[a]	4.6±0.4	3.9±0.3

Note. [a]P<0.05 compared to control or lipoate group; [b]P<0.02 compared to control or lipoate group; [c]P<0.001 compared to control or lipoate group; [d]P<0.05 compared to carboplatin group; [e]P<0.02 compared to carboplatin group; [f]P<0.001 compared to carboplatin group.

DISCUSSION

The present study addresses the changes in ABR relationship with the changes in cochlear GSH/GSSG ratio, antioxidant enzyme activities, enzyme protein expressions, and lipid peroxidation in carboplatin (256 mg/kg, i.p.) treated rats. These changes were partially attenuated with alpha-lipoic acid pretreatment. The data show that carboplatin significantly elevated the ABR thresholds at higher frequencies and significantly depleted GSH/GSSG ratio in the cochlea of the rat 4 days after treatment. Earlier study has shown ABR threshold changes and inner hair cell loss and type I auditory nerve in chinchillas after carboplatin administration[10]. However we did not observe hair cell loss in the cochlea of rats 4 days after carboplatin treatment (unpublished observations). We have previously reported the optimum dose and time of carboplatin-induced ototoxicity in a rat model[9,12]. These reports and observations suggest that central auditory system may likely be involved in carboplatin-induced ototoxicity. The data further indicate that alpha-lipoic acid partially prevents both the elevation of ABR thresholds and the depletion of cochlear GSH /GSSG ratio in rats treated with carboplatin four days post-treatment and provides partial protection to the cochlea. The depletion of GSH by buthionine sulfoximine (BSO) resulteds in the potentiation of ototoxicity of carboplatin[10], further suggesting the role of intracellular GSH in hearing process. Depletion of tissue GSH/GSSG ratio is a prime factor, which can impair the cell's defense against the toxic actions of ROS and may lead to peroxidative cell injury[31]. The decreased cochlear GSH/GSSG ratio, GR and GST activities after carboplatin suggest that GSSG is not reduced back to GSH due to impaired GR activity and also decreases metabolism via GST pathway. The increased cochlear MDA level suggests that membrane lipid peroxidation may be secondary to the inhibition of GR activity, and/or due to the generation of ROS by carboplatin[32]. Clinical studies have also shown that GSH is protective against toxicity of high dose carboplatin in cancer patients[4]. Alpha-lipoic acid has been shown to cause an increase in intracellular GSH in vitro as well as *in vivo*[33], which is an essential antioxidant for normal cochlear function. Interestingly, cochlear GSH /GSSG ratio in lipoate treated rats is above control value, indicating the interaction of lipoate with intra-cellular antioxidants. The pretreatment of rats with alpha-liboic acid partially prevented both the elevation of ABR thresholds and the depletion of cochlear GSH/GSSG ratio, suggesting that the ototoxic event correlated with oxidative stress and lipoate otoprotection is associated with elevation of cochlear GSH/GSSG ratio.

Carboplatin-induced ototoxicity may be a result of increased flux of ROS and enhanced membrane lipid peroxidation as a consequence of impaired cochlear antioxidant enzyme activities. The cochlear SOD, CAT, and GSH-Px activities in the carboplatin treated group were significantly inhibited as compared to the control group. The inhibition of antioxidant enzyme activity increases the endogenous superoxide anion, H_2O_2, and lipid peroxides, thus leading to Ca^{++} influx and pathological changes in cochlear cells[34-35]. The impaired antioxidant enzyme activity in the cochlea may result in the enhanced ROS-induced lipid peroxidation leading to ototoxicity. The inhibition of cochlear antioxidant enzymes and GR activities in carboplatin treated rats may be because of (1) direct binding of carboplatin to essential sulfhydryl groups of these enzymes, (2) depletion of copper and selenium essential for SOD and GSH-Px activities[36], (3) increased reactive oxygen species and organic peroxides inactivating antioxidant enzymes[37], and/or (4) depletion of GSH and NADPH essential for GSH-Px activity. The inhibition of antioxidant enzyme activities, GR and GST activities, and depletion of GSH/GSSG ratio, might be associated with the increase in ABR threshold. The inhibition of cochlear antioxidant enzyme and GR activities was partially recovered to the control levels by lipoate pretreatment. Interestingly, the inhibition of antioxidant enzyme activities (CuZn-SOD, CAT, GSH-Px and GST) was accompanied with depression of enzyme protein expressions 4 days after carboplatin administration in rats, indicating the influence of carboplatin on de novo synthesis of enzyme proteins in the cochlea. The antioxidant enzyme protein levels partially recovered in the carboplatin plus lipoate group compared to the carboplatin group. The Mn-SOD activity as well as protein expression increased after carboplatin and partially restored after lipoate pretreatment. The Mn-SOD activity is regulated by its biosynthesis, which is sensitive to tissue oxygenation, cytokines, tumor necrosis factor and corticosteroid hormones[38]. It is likely that carboplatin might have released these factors and thereby induced Mn-SOD activity in the cochlea. Moreover, ROS are generated after carboplatin administration and these are known to activate nuclear factor NF-kB and thereby induce transcription of Mn-SOD[39]. The present observations further support the role of oxidant/antioxidant systems in carboplatin-induced ototoxicity and otoprotective effects of the biological antioxidant alpha-lipoic acid in rats.

The exogenous thiol containing compounds or free radical scavenger amifostine and diethyldithiocarbamate

(DDTC) have been shown to attenuate carboplatin-induced toxicities specifically haematological toxicity in humans[13,16]. Sodium thiosulfate has been demonstrated to protect the carboplatin-induced ototoxicity in humans[8]. The chelating agent DDTC as a protective agent against cisplatin toxicity in humans has a number of unpleasant side effects such as numbness in the infusion arm, diaphoresis, chest discomfort, flushing, agitation and high systolic pressure[40]. This is the first report to show that alpha-lipoic acid partially protects carboplatin-induced ototoxicity in a rat model. It is suggested that some other mechanisms not directly relevant to oxidant/antioxidant may also likely be involved in otoprotection by lipoate against carboplatin-induced ototoxicity in rats.

In summary, carboplatin-induced ototoxicity is associated with a depletion of GSH/GSSG ratio, inhibition of antioxidant enzyme activities and protein expressions and increased lipid peroxidation in the cochlea, and is evidenced by elevated ABRs at higher frequencies. Alpha-lipoic acid administration before carboplatin treatment can offer partial protection against carboplatin-induced ototoxicity. The partial protection conferred by lipoate is related to preservation of the antioxidant system in the cochlea of rats.

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(*Received January* 6, 2004 *Accepted March* 29, 2005)