Research paper

Ebselen treatment reduces noise induced hearing loss via the mimicry and induction of glutathione peroxidase

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Abstract

Previous studies indicate that noise induced hearing loss (NIHL) involves a decrease in glutathione peroxidase (GPx) activity and a subsequent loss of outer hair cells (OHC). However, the cellular localization of this GPx decrease and the link to OHC loss are still poorly understood. In this report, we examined the cellular localization of GPx (GPx1, GPx 3 and GPx 4) in F-344 rat before and after noise exposure and after oral treatment with ebselen, a small molecule mimic of GPx activity. Results indicate that GPx1 is the major isoform within the cochlea and is highly expressed in cells of the organ of Corti, spiral ganglia, stria vascularis, and spiral ligament. Within 5 h of noise exposure (4 h at 113 dB, 4–16 kHz), significant OHC loss was already apparent in regions coincident with the 8– 16 kHz region of the cochlea. In addition, the stria vascularis exhibited significant edema or swelling and a decrease in GPx1 immuno-reactivity or fluorescent intensity. Treatment with ebselen (4 mg/kg p.o.) before and immediately after noise exposure reduced both OHC loss and the swelling of the stria vascularis typically observed within 5 h post-noise exposure. Interestingly, GPx1 levels increased in the stria vascularis after noise and ebselen treatment vs noise and vehicle-only treatment, and exceeded baseline no noise control levels. These data indicate that ebselen acts to prevent the acute loss of OHCs and reduces the acute swelling of the stria vascularis by two potential mechanisms: one, as a ROS/RNS scavenger through its intrinsic GPx activity, and two, as a stimulator of GPx1 expression or activity. This latter mechanism may be due to the preservation of endogenous GPx1 from ROS/RNS induced degradation and/or the stimulation of GPx1 expression or activity.
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1. Introduction

A common result of intense noise exposure is the development of a temporary threshold shift (TTS). Overtime, irreversible hearing loss can result producing a permanent threshold shift (PTS). Both TTS and PTS involve damage or loss of multiple cellular structures within the cochlea including hair cells, supporting cells, neurons of the spiral ganglia, and cells within the stria vascularis and spiral ligament. Generation or accumulation of reactive oxygen and/or nitrogen species (ROS/RNS) is thought to trigger a cascade of biochemical events that lead to cellular dysfunction and cell death in these cochlear structures (Yamane et al., 1995). In particular, lipid peroxidation has been observed within hours of noise exposure in these sensory and non-sensory cells (Ohinata et al., 2000).

Intense noise can also reduce glutathione (GSH) levels and/or increase the ratio of oxidized to reduced glutathione in the cochlea (Yamasoba et al., 1998). GSH is the primary substrate for glutathione peroxidase (GPx), an enzyme that catalyzes the ability of GSH to act as an anti-oxidant. Interestingly, GPx activity decreases following noise exposure (Ohlemiller et al., 1999). These data are consistent with the observation that deletion of the GPx1 gene confers increased susceptibility to noise damage and hearing loss (Ohlemiller et al., 2000).

Ebselen, a heterocyclic selenorganic, is a potent GPx mimic and neuroprotectant, and has strong activity against peroxynitrite (ONOO), a super ROS/RNS (Noguchi

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et al., 1992, 1994; Masumoto et al., 1996). Ebselen inhibits the mitochondrial permeability transition pore (Kowaltow-ski et al., 1998), reduces cytochrome C release and prevents nuclear damage in injured cells (Namura et al., 2001). Pre-clinical studies in rats and guinea pigs indicate that both the TTS and PTS induced by intense noise exposure can be reduced by ebselen when dosed at 4–10 mg/kg by oral gavage (Pourbakht and Yamasoba, 2003; Lynch et al., 2004). Because ebselen acts as a catalyst and is not con-sumed during redox reactions (Mu ̈ller et al., 1988), low oral doses may prove to be effective at preventing or treating human NIHL. Here we determined the major GPx isoform in the rat cochlea. Additionally, we quantified GPx1 immu-nofluorescense intensity in normal rat cochleae, and in cochleae from noise exposed rats with and without ebselen treatment.

2. Methods

2.1. Animals

Female F-344 rats, age 6 weeks, were purchased from Charles River Laboratories. Following 1 week of acclima-tion in our vivarium, isofluorane-anesthetized animals were uniquely identified by subcutaneuously implanted radio frequency transponders. Animals were housed in our SPF facility and routinely monitored for pathogens. All proto-cols were approved by the IACUC at Sound Pharmaceuti-cals, Inc., Seattle, WA.

Animals that were noise exposed and evaluated for hear-ing threshold shift were randomly assigned to control (vehicle-only) and ebselen-treated groups (3 or 14 d treat-ment), with n = 4 animals 8 ears/group. Animals analyzed for GPx expression were 10–12 week old female F-344 lit-termates born in house and divided into three groups: con-trol (no noise exposure, n = 3), vehicle-only (noise exposed, n = 3), and ebselen-treated (noise exposed, n = 3) for a total of nine cochleae analyzed from nine different animals.

2.2. Auditory assessment

Auditory function was evaluated by ABR threshold test-ing using methods previously described (Lynch et al., 2004). Only animals with normal baseline ABR thresholds at each test frequency (4, 8, 16, and 32 kHz) were entered into these studies. Threshold testing was repeated at 3, 6, 9, and 15 weeks post-noise exposure. Threshold shifts in dB were determined by subtracting baseline, or pre-noise threshold levels, from post-noise exposure thresholds for each test interval. Group mean threshold shifts were ana-lyzed for significant differences by ANOVA using the Stat-View™ (V.5.0) software package.

2.3. Noise exposure

Noise exposed animals were unanesthetized and housed in individual cages located 15 in. below the speaker and

exposed to the following stimulus conditions. The sound pressure level (SPL) was 113 dB SPL of 4–16 kHz one-octave band noise, for 4 h. Noise was generated and con-trolled with a custom software program (Beluga Software), then sent through an amplifier (Alesis RA100), a real-time processor (Tucker–Davis RP-2), a programmable attenua-tor (Tucker–Davis PA-5), and a high intensity horn-driver speaker (Visaton HTH 8.7). A Quest QC-20 sound level meter with a Bruel and Kjaer Type 4134 microphone was used to calibrate noise levels before and after the 4 h expo-sure. The speaker system was operated in a ventilated sound chamber (Industrial Acoustics Company, model IAC-2).

2.4. Ebselen (SPI-1005) formulation and dosing

Ebselen (>99% pure by HPLC, Rhodia Pharma Solu-tions, custom synthesis for SPI) was dissolved in pure DMSO at 10 mg/ml. The 10 mg/ml stock solution (SPI-1005) was diluted in sterile normal saline immediately prior to use for oral delivery by gavage (4 mg/kg p.o.). Where appropriate, animals were dosed twice daily 1 d before noise, 1 h pre-noise, and 1 h post-noise. Control animals received an equal volume of vehicle with matching final concentration of DMSO on the same dosing schedule as SPI-1005 treated animals.

2.5. Analysis of GPx1, GPx3, and GPx4 expression

Cochleae from 1 to 2 month old rats were collected and fixed with 4% paraformaldehyde (PFA) for 2 h, then stored in PBS at 4 °C for up to 4 weeks. After decalcification for 1 week with 0.5 M EDTA, pH 7.4, cochleae were cryopro-tected with a 30% sucrose/phosphate buffered saline (PBS) solution, embedded in Optimal Cutting Temperature (OCT) medium and frozen at 80 °C. The cochlear sec-tions were made along the mid-modiolar axis at 6 μm thickness and adhered to glass slides and kept at 80 °C until further processing. The frozen sections were brought to RT and blocked for 1 h with a 10% normal goat serum solution and incubated with a rabbit anti-GPx1, 3, or 4 at 1:200 (Lab Frontier, S. Korea) at 4 °C overnight. After three 5 min washes with PBS, goat anti-rabbit-FITC 1:200 was added for 1 h at RT. After further PBS washes, the sections were coverslipped with a DAPI containing counterstain to identify cell nuclei.

2.6. Determination of GPx1 fluorescent intensity before and after noise exposure

Cochleae from rats exposed to noise with or without SPI-1005 treatment, and no noise controls, were collected at 5 h post-noise exposure and immediately fixed with 4% PFA for 2 h. After decalcification cochleae were embedded in OCT media and frozen, and 6 μm mid-modiolar sections from each group were placed on the same slide. The sec-tions were incubated with rabbit anti-GPx1 at 1:200 over-night at 4 °C. After three 5 min washes with PBS, goat

anti-rabbit-FITC 1:200 was added for 1 h at RT. After another series of PBS washes, a coverslip was placed on the sections with DAPI in the mounting media. Images of cochlear sections were captured on a Princeton Instruments CCD camera mounted to a Nikon E-800 epifluorescent microscope. Digital images of the stria vascularis and spiral ligament were captured at a 1 s fixed exposure with a 20· objective. Images were further analyzed using Metamorph⁻ V6.2.3.6 software to determine the fluorescent intensity (FI). The stria vascularis and spiral ligament regions were traced to determine their area in lm^2 using Metamorph. The Relative Fluorescent Units (RFU) were then determined for each structure. Statistical evaluations of RFU were made between treatment groups using an ANOVA (StatView⁻ V5.0). RFU measurements (Mean ± SEM) were analyzed from approximately 16 basal turn mid-modiolar sections per cochlea. Sections were taken from the left cochlea of three rats per treatment group. Sec-tions per group: n = 48 (no noise controls), 49 (noise + vehicle), and 54 (noise + SPI-1005).

2.7. Cytocochleogram analysis

At 15 weeks post-noise exposure, animals were sacrificed by CO_2 asphixiation to cardiac arrest to allow for collection of cochleae. Following animal sacrifice, a small hole was made at the apex of the cochlea and 4% paraformaldehyde was slowly perfused through the round window. Temporal bones were removed and immersed in the same fixative for 1 h. Samples were stored at 4 LC in PBS for 1–4 weeks. Each cochlea was dissected, the tectorial membrane was removed, and the tissue was decalcified with 0.5 M EDTA for 30 minutes. Cochlear tissue was then labeled with 1 lg/mL FITC-phalloidin in PBS for 1 h. Microdissected turns of sensory epithelia from the whole cochlea were cut into three pieces (apical turn, basal turn, and hook region) on a microscope slide in mounting media containing Diami-dino-2-phenylindole (DAPI). Digital images of cochleae were captured via a CCD camera (Princeton Instruments, 1300-Y) mounted on an epi-fluorescent microscope (Nikon, E800). Whole cochlear turn images were assembled from a series of digital images using Adobe Photoshop software. Missing OHCs were defined as the absence of a DAPI stained nucleus and phalloidin-FITC labeled stereocilia in a region of Corti's organ where one would normally expect an OHC to be present. The number of missing OHCs along each 100-lm length was counted, and the percentage of OHC loss was calculated for each region. Typically, a total of 37–39 OHCs and 11–12 IHCs were present in 100 lm of a normal rat cochlea in the basal turn.

3. Results

3.1. GPx expression

We determined the expression pattern of GPx1, GPx3, and GPx4 in fixed rat cochleae from 10–12 week old litter-mates (Fig. 1). In the organ of Corti, GPx1 expression appeared in both inner and outer hair cells and in all supporting cell types. In supporting cells, especially Deiter's cells, GPx1 labeling appeared in both the cytoplasm and nuclei. In the spiral ganglia, GPx1 labeling appeared in several cell types including type I neurons. Here, the nuclei of type I neurons appeared negative when compared to the cytoplasm. GPx1 labeling appeared in all cell types of the stria vascularis and in many of the cell types of the spiral ligament, including type III and type IV fibrocytes. In the organ of Corti, spiral ganglia, stria vascularis and spiral ligament, GPx3 and GPx4 labeling was absent relative to no primary antibody labeled controls. Results for GPx1, GPx3, and GPx4 labeling in mouse cochlea are equivalent to those described here for rat cochlea (data not shown).

3.2. GPx1 expression following noise exposure

We examined and quantified the acute changes in strial area and GPx1 expression following noise exposure using immunofluorescence in SPI-1005 treated rats, vehicle-only treated rats, and in no noise controls (Figs. 2 and 3). Strial images from normal rat cochlea labeled with GPx1 had a mean of 297 RFU/lm^2 (SEM = 8.5, n = 48). Vehicle trea-ted noise exposed rat cochlea had a mean GPx1 labeling of 271 RFU (SEM = 8.1, n = 49), while noise exposed rats treated with SPI-1005 had a mean GPx1 labeling of 324 RFU (SEM = 10.3, n = 54) per strial image. This corresponds to a decrease of 8.8% in GPx1 area weighted intensity for vehicle treated rats (p = 0.052) and an increase of 9.1% GPx1 area weighted intensity for SPI-1005 treated rats (p < 0.05), relative to no noise controls.

Similar GPx1 fluorescent intensity and area analyses were performed for acute changes in spiral ligament following noise exposure in SPI-1005 treated rats, vehicle-only treated rats, and in no noise controls. Spiral ligament images from normal rat cochlea labeled with GPx1 had a mean of 215 RFU/lm^2 (SEM = 5.9, n = 39). Vehicle trea-ted noise exposed rat cochlea had a mean GPx1 labeling of 201 RFU (SEM = 4.4, n = 45), while noise exposed rats treated with SPI-1005 had a mean GPx1 labeling of 235 RFU (SEM = 7.2, n = 48) per spiral ligament image. This corresponds to a decrease of 6.5% in GPx1 area weighted intensity for vehicle treated rats (n.s.) and an increase of 9.3% GPx1 area weighted intensity for SPI-1005 treated rats (p < 0.05), relative to no noise controls.

3.3. Auditory analysis

ABR threshold shifts were determined at time points up to 15 weeks post-noise exposure. Significantly reduced ABR threshold shifts at all frequencies tested were observed in treated groups receiving either 3 d or 14 d bid dosing of 4 mg/kg SPI-1005 when compared to noise exposed vehicle treated controls (Fig. 4A). These results indicate that extending SPI-1005 treatment beyond 1 d
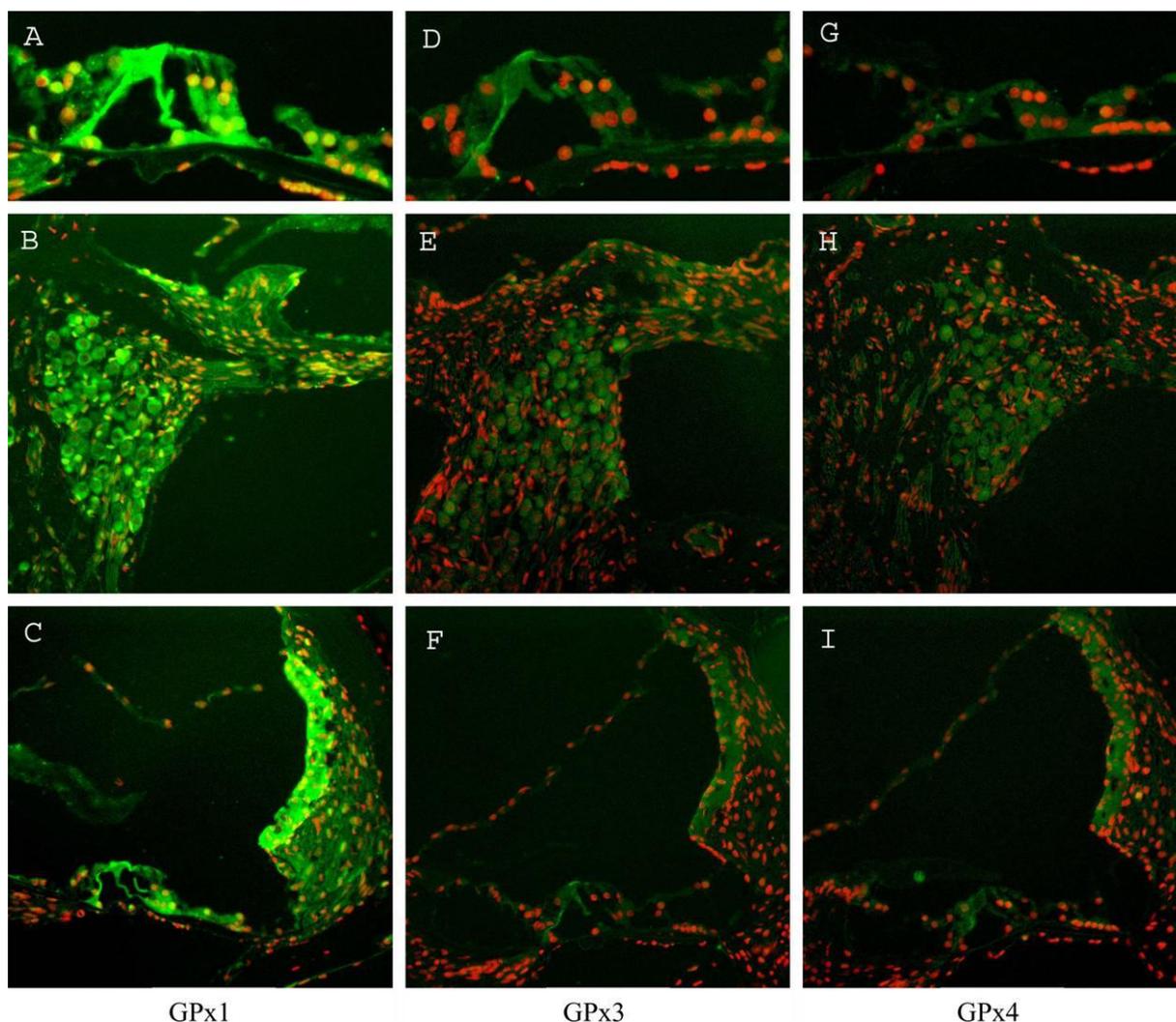
Fig. 1. Evaluation of GPx1, GPx3, and GPx4 expression in the normal adult rat cochlea. High intensity labeling for GPx1 (green) was observed for hair cells and supporting cells in the organ of Corti (A), the spiral ganglion neurons (B), and the stria vascularis and spiral ligament (C). Notably, the supporting cells consistently have nuclear labeling for GPx1 while OHCs do not exhibit nuclear GPx1 labeling (red nuclei). Low intensity labeling for GPx3 was observed in the organ of Corti (D), spiral ganglion neurons (E), stria vascularis and spiral ligament (F). Low intensity labeling for GPx4 was observed in the organ of Corti (G), spiral ganglion neurons (H), stria vascularis and spiral ligament (I).

after noise (i.e., 14 d dosing group) increases the level of otoprotection. A significant difference in ABR threshold shifts was observed at 15 weeks post-noise (Fig. 4B) expo-sure between the 14 d treatment group and the 3 d treat-ment group ($p < 0.05$).

3.4. Cytocochleogram analysis

Evaluation of OHC loss at 15 weeks post-noise exposure in vehicle treated vs SPI-1005 treated rat cochlea (3 d and 14 d dosing) showed reduced OHC loss in both SPI-1005 treated groups with the best effect following 14 d of SPI-1005 treatment (Fig. 5). The reduction in OHC loss for the SPI-1005 treated groups is consistent with lower ABR threshold shifts relative to vehicle treated controls. In com-parison to the control group ($n = 7$), the 3 d treatment group had a 58% reduction ($p < 0.01$, $n = 8$), and the

14 d treatment group had a 72% reduction ($p < 0.001$, $n = 8$) in total OHC loss.

4. Discussion

Recently, four reports have shown that ebselen reduces NIHL following low oral dosing (Pourbakht and Yama-soba, 2003; Lynch et al., 2004; Lynch and Kil, 2005; Yama-soba et al., 2005). A significant decrease in PTS has been reported in both guinea pig and rat that correlated to a sig-nificant reduction in OHC loss. Similarly, a decrease in TTS was associated with a reduction in afferent dendrite swelling of the spiral ganglia. Here, we report that GPx1 immunofluorescense intensity per unit area in the stria vascularis decreases acutely following noise exposure, and this reduction can be inhibited by ebselen treatment. The mechanism of action of ebselen in the stria vascularis and
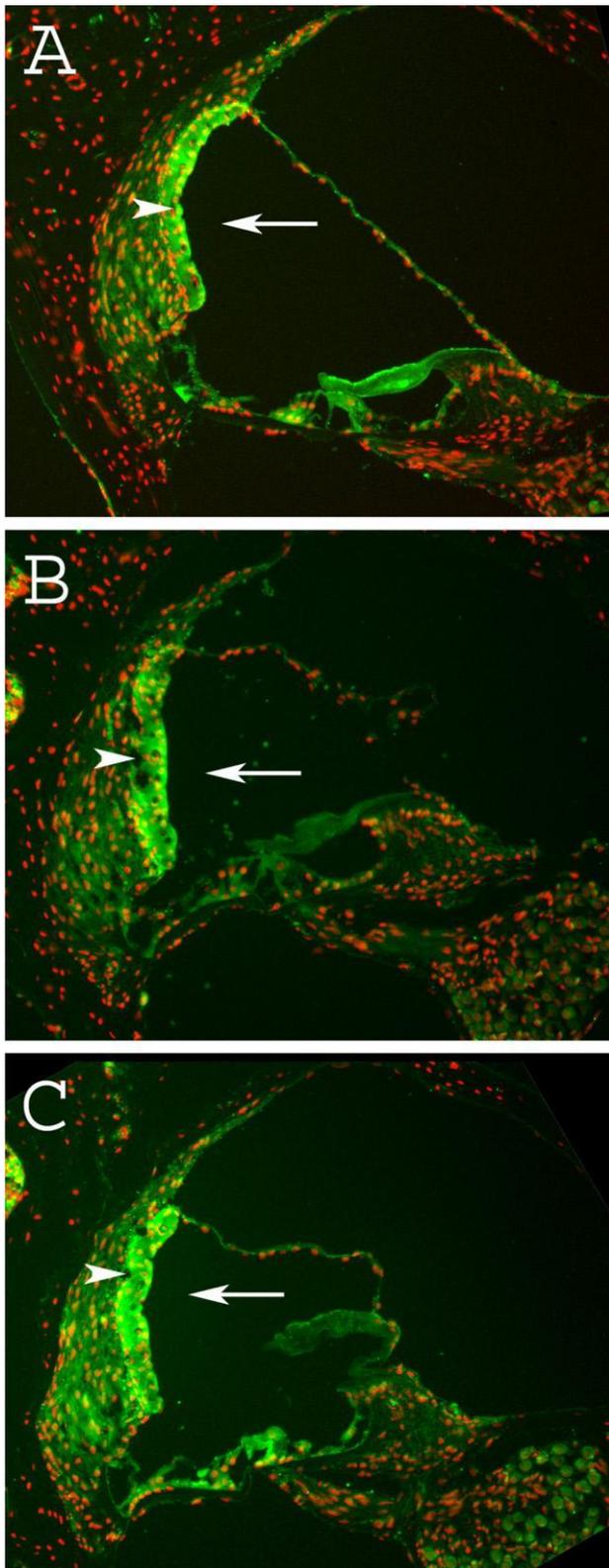
Fig. 2. Changes in GPx1 immunoreactivities 5 h after noise exposure (113 dB, 4–16 kHz, 4 h). GPx1 labeling in the cochlea of non-noise exposed rats (A). After noise exposure in rats treated with vehicle, the stria vascular is appears edematous with a decreased level of GPx1 immuno-fluorescence (B). At the same time point, the stria vascularis from rats treated with SPI-1005 are less edematous and exhibit an increased level of GPx1 immunolabeling (C). Arrowheads indicate the spiral ligament. Arrows indicate the stria vascularis.

spiral ligament appears to involve the induction or preser-vation of GPx expression. It is unclear whether the con-comitant decrease in strial edema is dependent upon the increase in endogenous GPx expression or the effect of ebselen on a specific cell type within the stria vascularis. In normal cells, GPx functions at near maximal levels. In myocardial cells, ebselen prevents the lipid peroxidation and subsequent LDH release typically observed following hydrogen peroxide injury. In that model system, ebselen was found to induce glutathione reductase (GR) activity, but not GPx activity (Hoshida et al., 1997). Therefore, it was unexpected that low oral doses of ebselen could stim-ulate GPx levels in the stria vascularis and spiral ligament.

Our results are consistent with a previous report that demonstrates an acute swelling of the stria vascularis fol-lowing noise exposure in mice (Hirose and Liberman, 2003). In this study, pathologic changes appeared as large intercellular spaces and type II fibrocyte degeneration as early as 8 h after noise exposure. These changes were asso-ciated with a decrease in endocochlear potential and con-comitant TTS at 24 h. It is becoming more apparent that acute noise exposure can result in pathologies that effect sensory and non-sensory structures in the cochlea that are also affected in age related hearing loss.

In general, ebselen is capable of reducing oxidative stress levels in various cell types potentially through a variety of mechanisms. Intense noise exposure can lead to increased oxidative stress causing OHC loss via activation of apopto-tic and necrotic pathways. Noise exposure causes the release of cytochrome c from mitochondria in both apopto-tic and necrotic cells. The release of cytochrome c in a sub-population of OHCs takes place early in the cell death process, prior to any outward signs of apoptosis or necrosis (Nicotera et al., 2003). In other studies, ebselen has been shown to inhibit these specific proapoptotic events (Kowal-towski et al., 1998; Namura et al., 2001).

Ebselen is multi-functional in its otoprotective activity with the ability to inhibit the pro-oxidative enzyme iNOS (Zembowicz et al., 1993; Hattori et al., 1996), and to mimic the anti-oxidative enzyme GPx (Fig. 6). Nitric oxide syn-thase is induced in the cochlea following noise exposure (Shi et al., 2002) and appears robust in cells of the stria vascularis (Shi and Nuttall, 2003). Ebselen has also been shown to lower IL-6 plasma levels after focal cerebral ischemia in rats (Gladilin et al., 2000). Recently, IL-6 expression was found to be induced within the spiral liga-ment and stria vascularis of noise exposed rats (Fujioka et al., 2006).

Ebselen has been described as having the properties of a thioredoxin reductase (TrxR) peroxidase (Zhao et al., 2002), a dehydroascorbate reductase and a thioltransferase (Jung et al., 2002), an anti-inflammatory compound (Wen-del et al., 1984; Bosch-Morell et al., 2002), and an anti-apoptotic compound (Namura et al., 2001; Ramakrishnan et al., 1996). In the auditory system, ebselen may exert its protective effects in part through the induction of endoge-nous GPx activity. Thus, the precise biochemical mecha-
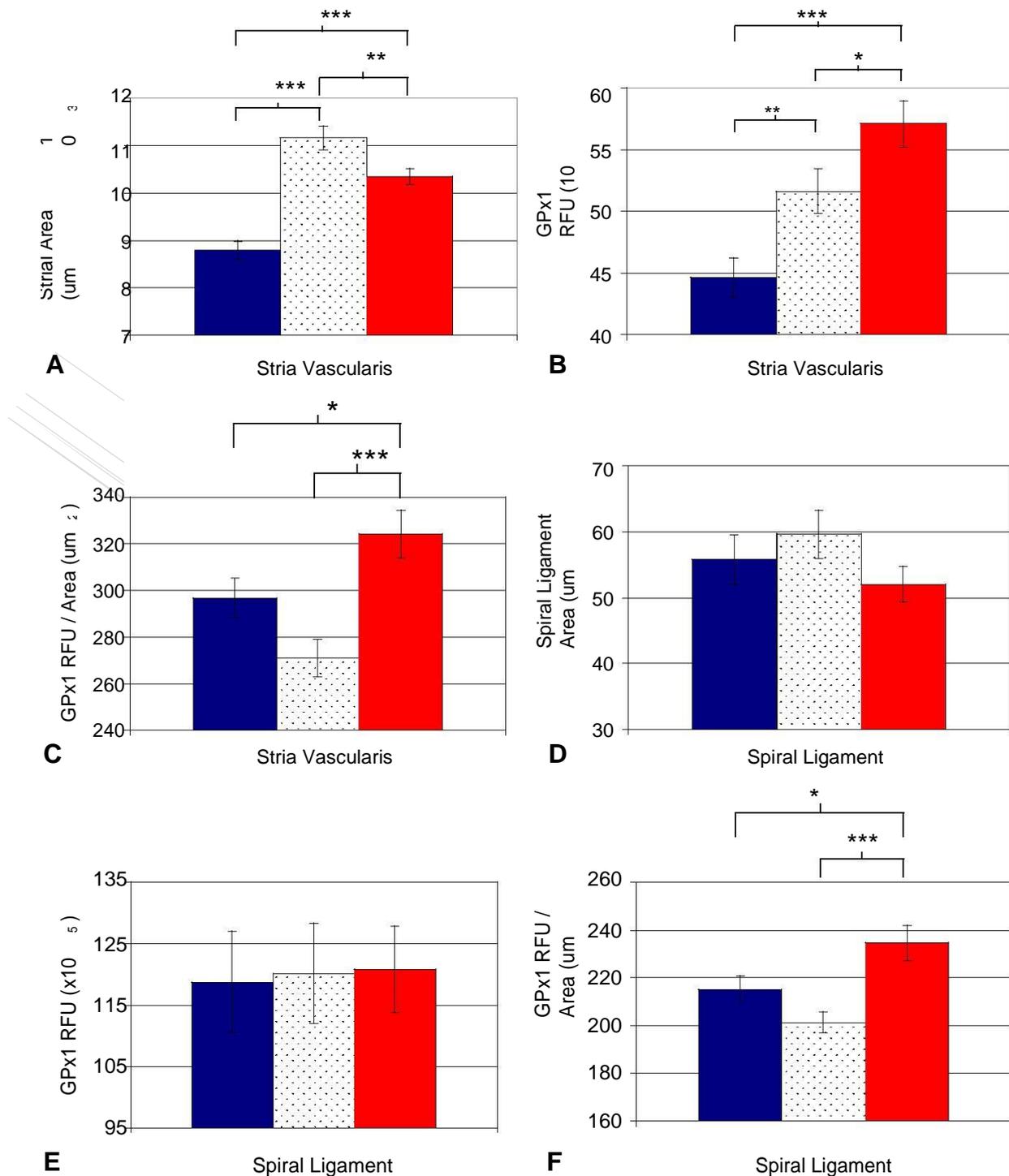
Fig. 3. Analysis of GPx1 immunolabeling in the stria vascularis (A–C) and spiral ligament (D–F) at 5 h post-noise exposure (blue bars = normal rats; gray bars = noise exposed vehicle treated rats; red bars = noise exposed SPI-1005 treated rats). Area (A, D), fluorescent intensity (B, E), and relative fluorescent intensity per unit area (C, F) are graphically represented. Strial area increases in noise exposed rats with vehicle, but is significantly less increased with SPI-1005 treatment ($p < 0.01$) (A). GPx1 fluorescent intensity increases following noise and is significantly greater with SPI-1005 treatment ($p < 0.05$) (B). Strial GPx1 fluorescent intensity/area is significantly greater with SPI-1005 treatment vs vehicle treatment ($p < 0.001$) (C). Spiral ligament area changes non-significantly in vehicle treated and SPI-1005 treated rats ($p > 0.05$) (D). GPx1 fluorescent intensity in spiral ligament is similar in all groups (E). Spiral ligament GPx1 fluorescent/area is significantly greater with SPI-1005 treatment vs vehicle treatment ($p < 0.001$) (F). ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

nism(s) of ebselen mediated otoprotection in the cochlea of noise exposed animals may be multi-factorial involving both sensory and non-sensory structures. When compared to other otoprotective agents (reviewed in Lynch and Kil, 2005), ebselen is unique in its ability to prevent both the TTS and PTS induced by noise with low oral doses. Ebse-
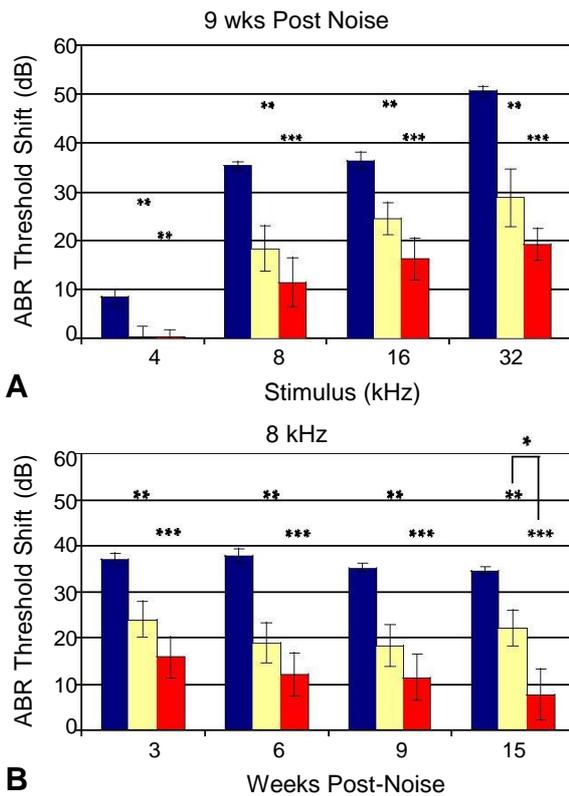
A

B

Fig. 4. Mean ABR threshold shift at 4, 8, 16, and 32 kHz for control (blue), 3-day (yellow) and 14-day (red) SPI-1005 (ebselen) treated groups tested at 9 weeks post-noise exposure (A), and mean threshold shift at 8 kHz tested at 3, 6, 9, and 15 weeks post-noise exposure (B). Rats were exposed for 4 h to 113 dB SPL OBN centered at 8 kHz. Treatment began 1 d prior to noise exposure. SPI-1005 was dosed orally, twice daily, at 4 mg/kg for the durations indicated (n = 8 ears/group, SEM shown, [*] p < 0.05, [**] p < 0.01, [***] p < 0.001).
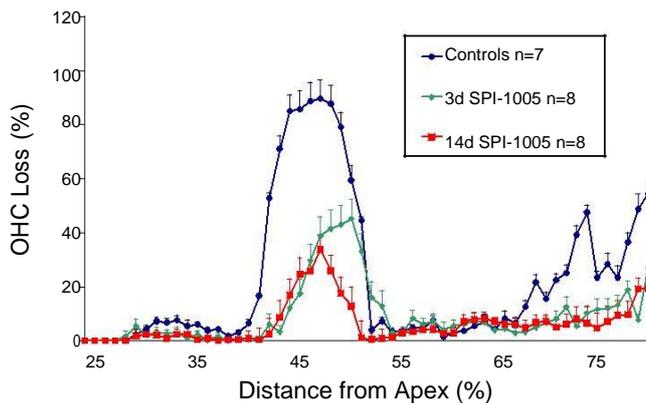

Fig. 5. Cytocochleogram at 15 weeks post-noise exposure in rats. Percent outer hair cell loss is indicated on the y-axis. Percent distance from the apex is indicated on the x-axis. The upper limit of quantification occurred at 80% distance from the apex and did not include the hook region. Cochlea were analyzed in 100 lm lengths, and the level of OHC loss indicated as a single point on the x-axis (error bars shown are SEM).

len-treated rats show reduced ABR threshold shifts and OHC loss out to 15 weeks post-noise exposure. In addition, ebselen appears to be unique in that it reduces the patho-
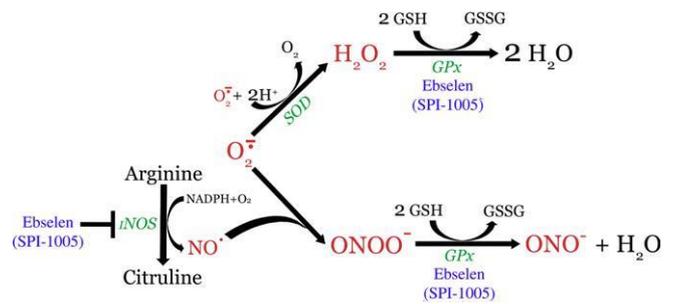

Fig. 6. Proposed mechanism of action of Ebselen in preventing and treating noise induced hearing loss. The major ROS/RNS species generated by noise are indicated in red. Anti-oxidant and pro-oxidant enzymes are indicated in green. Ebselen's ability to mimic GPx or inhibit iNOS is indicated in blue.

logic changes in both sensory and non-sensory structures through anti-oxidative, anti-apoptotic, and anti-inflamma-tory pathways. For these reasons, ebselen may prove to be an effective compound to prevent and treat age related hearing loss, a disease that is thought to affect both sensory and non-sensory structures of the cochlea including hair cells, spiral ganglia neurons, and stria vascularis (Schuknecht and Gacek, 1993).

Acknowledgements

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