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## Expression pattern of oxidative stress and antioxidant defense-related genes in the aging Fischer 344/NHsd rat cochlea

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### Abstract

The biological mechanisms that give rise to age-related hearing loss (ARHL) are still poorly understood. However, there is growing recognition that oxidative stress may be an important factor. To address this issue, we measured the changes in the expression of cochlear oxidative stress and antioxidant defense-related genes in young (2 months old), middle-aged (12 months old), and old (21–25 months old) Fischer 344/NHsd (F344/NHsd) rats and compared gene expression changes with ARHL. A quantitative real-time reverse transcription polymerase chain reaction array revealed a significant age-related downregulation of only 1 gene, stearoyl-coenzyme A desaturase 1, and upregulation of 12 genes: 24-dehydrocholesterol reductase; amino adipate-semialdehyde synthase; cytoglobin; dual oxidase 2; glutathione peroxidase 3; glutathione peroxidase 6; glutathione S-transferase, kappa 1; glutathione reductase; nicotinamide adenine dinucleotide phosphate (NAD(P)H) dehydrogenase, quinone 1; solute carrier Family 38, Member 5; thioredoxin interacting protein; and vimentin. Statistical analyses revealed significant correlations between gene expression and auditory function in 8 genes. Our results identified specific subsets of oxidative stress genes that appear to play an important role in ARHL in the Fischer 344/NHsd rat.

### Keywords

Age-related hearing loss; Presbycusis; Cochlea; Fischer 344; Antioxidant; Reactive oxygen species; Oxidative stress; Rat; Genes; Scd1; Dhcr24; Aass; Cygb; Duox2; Gpx3; Gpx6; Gstk1; Gsr; Nqo1; Slc38a5; Txnip; Vim

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#### Disclosure statement

The authors disclose no conflicts of interest.

All procedures involving use and care of the animals were reviewed and approved by the State University of New York at Buffalo Institutional Animal Care and Use Committee.

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## 1. Introduction

Reactive oxygen species (ROS) that are generated during normal metabolism can be effectively eliminated by cellular antioxidant defense mechanisms. Excessive production of ROS, however, overwhelms the antioxidant defense system, leading to oxidative stress and tissue damage as seen in noise-induced hearing loss (Henderson et al., 2006). Increased free radical activity and oxidative stress have been proposed as key determinants of the aging process (Harman, 1956; Sohal and Weindruch, 1996) and tissue degeneration in aging mouse cochleae (Jiang et al., 2007; Staecker et al., 2001).

Mitochondria in particular are considered to be a major source of ROS overproduction in aging tissues (Harman, 1972), and excessive ROS could damage mitochondrial structures and molecules, leading to DNA mutations or deletions. Emerging evidence shows mitochondrial damage in age-related hearing loss (ARHL) in humans (Markaryan et al., 2008) and in various animal models, including DBA/2J and Polg(D257A) knockin mice (Someya et al., 2007; Yamasoba et al., 2007), and Fisher 344/NHsd (F344/NHsd) rats (Seidman et al., 2000; Yin et al., 2007).

The F344/NHsd rat exhibits increased mitochondrial DNA deletions in the cochlea at 6 and 9 months of age (Seidman et al., 2000; Yin et al., 2007). The accumulation of mitochondrial DNA deletions or mutations during aging is thought to impair mitochondrial function, resulting in the buildup of cochlear oxidative stress and consequent cochlear degeneration and ARHL (Fig. 1).

The antioxidant defense system is our natural defense against oxidative stress. To understand the role of antioxidant enzymes in the aging degeneration, 2 studies have examined changes in the level of cochlear antioxidant enzyme activity in aging F344/NHsd cochlea (Coling et al., 2009; Lautermann et al., 1997). Compared with 3-month-old rats, 24-month-old F344/NHsd rats exhibit decreased glutathione levels in the auditory nerve. However, enzyme activities of glutathione reductase (Gsr) and glutathione S-transferase (Gst) remained unchanged in the cochlear sensory epithelium (CSE), the lateral wall (LW), auditory nerve, and the vestibular system (Lautermann et al., 1997). Mean glutathione peroxidase (Gpx) activity in the CSE, LW, and auditory nerve increased in the 24-month-old F344/NHsd rats, but the increase was not statistically significant. Another study (Coling et al., 2009) reported a statistically significant increase in Gpx in the LW, but not in the auditory nerve or CSE, suggesting that age-related changes in activities of these enzymes appear to be tissue specific.

Changes in antioxidant capacity are likely to be caused by expression changes in antioxidant enzymes and molecules. So far, only 1 study has examined age-related changes in antioxidant enzyme gene expression in F344/NHsd rats (Chen and Ruan, 2009). However, this study revealed no significant changes in Cu/Zn-superoxide dismutase (Sod1), Mn-Sod (Sod2), catalase, or Gpx1 in either the auditory cortex or the cochleae. Therefore, a comprehensive analysis of expression patterns of the genes related to oxidative stress and antioxidant defense is necessary.

Human ARHL or presbycusis has been categorized into 6 types (Schuknecht and Gacek, 1993): (1) sensory presbycusis, caused primarily by loss of outer hair cells (OHCs) in the basal end of the cochlea; (2) neural presbycusis, characterized by degeneration of cochlear neurons; (3) stria or metabolic presbycusis, with degeneration in stria vascularis; (4) cochlear conductive presbycusis, hypothetically associated with changes in the stiffness properties of the basilar membrane; (5) mixed presbycusis, a combination of different pathologies; and (6) indeterminate presbycusis, with no obvious cellular pathology, indicating a possibility of impaired cellular function rather than cellular attrition.

To investigate these types of hearing loss, several animal species such as mice, rats, chinchillas, gerbils, and guinea pigs, have been used. Each animal model mimics distinct types of human presbycusis. For example, mice are reported to mimic all types of presbycusis, except for cochlear conductive and indeterminate presbycusis; and rats mimic all types, except for cochlear conductive presbycusis (see Ohlemiller, 2006, 2009 and Syka, 2010 for reviews).

The F344 inbred albino rat, with a median life span of 28–31 months (Chesky and Rockstein, 1976; Rao and Boorman, 1990), is widely used as an animal model of aging and ARHL. Despite the extensive use of the F344, genetic contributions to ARHL in the F344 are still not well understood and need to be investigated. The F344 was originally bred as a natural cancer model at Columbia University (Festing, 1979; Tanaka et al., 2000). Later, the F344 that was brought to the National Cancer Institute was denoted as F344/N and distributed in the USA. The substrain that went to the Charles River Laboratory was denoted as F344/Du and used in Europe for research.

Both the F344/NHsd and F344/DuCr1, which are used in ARHL research, initially develop high-frequency hearing loss that gradually progresses to the low-frequencies later in their lives (Bielefeld et al., 2008; Popelar et al., 2006). The deterioration of auditory function in F344/DuCr1 rats is accompanied by pathological changes in the cochlea, including the loss of OHCs, decreased immunostaining of collagen, and fibrocytes in spiral ligament. Possible changes in the collagen of the tympanic membrane were also suggested (Buckiova et al., 2006, 2007; Popelar et al., 2006; Syka, 2010). These investigators also reported high numbers of apoptotic cells in the stria vascularis in 20- to 24-month-old F344/DuCr1 rats, suggesting a possibility of abnormal endocochlear potentials (EPs). In contrast, F344/NHsd rats have normal endocochlear potentials at the same age. The differences in cochlear pathologies between the 2 substrains are difficult to assess because the 2 substrains have not been studied simultaneously in 1 laboratory.

The F344/NHsd substrain develops hearing loss starting in the 20–40 kHz range between 9 and 12 months of age, extending down to 5–10 kHz by 18 months of age (Bielefeld et al., 2008). Age-related decreases in distortion product otoacoustic emission (DPOAE) amplitudes were observed in both substrains. Cochlear pathologies in F344/NHsd rats include the loss of OHCs (Bielefeld et al., 2008; Hu et al., 2008) and spiral ganglion cells (SGCs) in the basal and middle turns (Keithley et al., 1992).

In 20- to 27-month-old F344/NHsd rats, OHCs were present in the middle to upper basal turn, but surprisingly, DPOAE amplitudes in these regions were reduced in the aged F344/NHsd rats. These results suggest that functional impairments occur long before OHC degeneration (Bielefeld et al., 2008; Chen et al., 2009). In addition, a reduction in DPOAE amplitudes in the aged F344/NHsd rats was associated with reduced immunolabeling of the OHC electromotility protein, prestin (Chen et al., 2009), which is believed to underlie cochlear amplification and DPOAE generation (Liberman et al., 2002; Zheng et al., 2000). The reduced prestin immunolabeling led to the hypothesis that the F344/NHsd rat is a model of indeterminate presbycusis because hearing loss results from OHC malfunction rather than OHC loss.

While the physiological and pathological changes in aging F344/NHsd rats have been relatively well documented, the molecular mechanisms responsible for these changes are still not clear. Given the importance of oxidative imbalances in ARHL, we designed the study to screen genes related to oxidative stress and antioxidant defenses for their involvement in the cochlear aging process. Gene expression changes in cochlear tissue were measured at several different ages in F344/NHsd rats using a quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR) array containing 84 relevant genes. In addition, the gene expression changes were tested for correlation with changes in hearing function as assessed with DPOAE and the auditory brainstem response (ABR). The current study will serve as the first phase to identify significant cochlear genes in oxidative stress and antioxidant defense pathway in relation to ARHL. Our results recognized specific subsets of oxidative stress genes that appear to play an important role in ARHL in the F344/NHsd rat.

## 2. Methods

### 2.1. Subjects

Three groups of F344/NHsd inbred male albino rats, young (2 months old,  $n = 10$ ), middle-aged (12 months old,  $n = 8$ ), and old (21–25 months old,  $n = 10$ ), were obtained from Harlan Laboratories, Inc., National Institute of Aging (Bethesda, MD, USA, for the old age group) and Taconic Farms, Inc. (Germantown, NY, USA, for the young and middle age groups). All procedures involving use and care of the animals were reviewed and approved by the State University of New York at Buffalo Institutional Animal Care and Use Committee.

### 2.2. ABR test

The animals were anesthetized with inhalant isoflurane (4% for induction, 1.5% for maintenance, 1 L per minute O<sub>2</sub> flow rate). ABR recordings were performed in a soundproof booth. Subcutaneous needle electrodes were placed at the vertex (noninverting), below the left pinna (inverting), and behind the shoulder blade (ground). During the ABR recordings, the animals were placed on a homeothermic blanket (Model 50–7053-F, Harvard Apparatus, Holliston, MA, USA) to maintain body temperature. Test stimuli consisted of alternating phase tone bursts at frequencies of 5, 10, 15, 20, 30, and 40 kHz. Signals were generated using Tucker Davis Technologies (TDT, Gainesville, FL, USA) SigGen32 software. Each tone burst (1-ms duration) was gated with a Blackmann window that had a

0.5 ms rise/fall time with no plateau. Stimuli were presented at a rate of 21 per second. Signals were routed to a Dome tweeter (model FT28D, Fostex, Tokyo, Japan) positioned at 0 degrees azimuth, 17 cm from the vertex of each animal's head. The signals were calibrated before the testing sessions. The animals' evoked responses were amplified with a gain of 50,000 times, using a TDT Headstage-4 bioamplifier and bandpass filtered from 100 to 3000 Hz. Responses to 300 sweeps were averaged at each stimulus level using TDT BioSig32 software. The level of the signals was decreased in 5-dB steps from 90 dB sound pressure level (SPL) to 10 dB below that of the lowest level that evoked a detectable and repeatable response. Threshold was defined as the lowest level at which a detectable response could be elicited and could be repeated.

### 2.3. DPOAE

While the rats were under isoflurane anesthesia, DPOAEs were recorded using a SmartDPOAE System (Version 4.53, Intelligent Hearing System, Miami, FL, USA) in a sound-attenuating chamber. During the DPOAE recording, the animals were placed on a homoeothermic blanket. Otosopic examinations were performed prior to testing to identify the presence of any cerumen. An earpiece attached to a microphone (Etymotic10B, Etymotic Research, Inc., Elk Grove Village, IL, USA) and 2 sound delivery tubes were inserted into the ear canal. Two IHS-3738 high-frequency transducers (Intelligent Hearing System) were used to deliver the 2 primary tones,  $F_1$  and  $F_2$ , to the ear canal via the 2 flexible tubes connected to the earpiece. The  $F_2/F_1$  ratio was set at 1.2, with 10 dB difference between  $F_1$  and  $F_2$  intensities ( $L_1$  and  $L_2$ , respectively). The output of the microphone was fed to the input of the Smart DPOAE system, digitized, and evaluated using the system software. Cubic DPOAEs were measured at the frequency of  $2F_1-F_2$ . The intensity of  $F_1$  was decreased in 5-dB steps from 70 to 25 dB SPL. At least 2 runs were recorded from each ear, and the average amplitude from the 2 ears, using the run with the higher amplitude in the 2 runs was used for final analyses.

### 2.4. Cochleogram

Cytochleograms were constructed by counting the number of OHCs in 9 cochleae ( $n = 3$  per group). The cochleae were fixed with 10% buffered formalin for 2 hours and then transferred to phosphate buffered saline (PBS) for dissection. The dissected sensory epithelium was permeabilized in 0.5% Triton X-100 in PBS for 30 minutes and placed in Blocker Casein in PBS (Thermo Fisher Scientific, Inc., Rockford, IL, USA) for 2 hours. The specimens were incubated in antiprestin antibody (C-16, goat polyclonal IgG, catalog number sc-22694, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at a concentration of 0.4  $\mu\text{g}/\text{mL}$  in PBS containing 5 mg/mL bovine serum albumin (PBSB) overnight at 4 °C and then in the fluorescein-conjugated secondary antibody (donkey antigoat Alexa 488, diluted 1:500 in PBSB, Invitrogen Corp., Carlsbad, CA, USA) for 1 hour at room temperature. The tissues were colabeled with propidium iodide (PI, 5  $\mu\text{g}/\text{mL}$  in PBS containing 0.25% Triton-X-100 and 1% bovine serum albumin, Invitrogen Corp.) for 5 minutes for nuclear staining. The stained specimens were mounted on slides with ProLong gold antifade reagent (Invitrogen Corp.). The OHCs were judged as absent if both prestin and propidium iodide fluorescent signals were undetectable.

## 2.5. qRT-PCR array

The Rat Oxidative Stress and Antioxidant Defense RT<sup>2</sup> Profiler PCR Array (catalog number PARN-065A, SABiosciences Corp., Frederick, MD, USA) was used to measure the messenger ribonucleic acid (mRNA) expression levels of oxidative stress and antioxidant defense-related genes in the cochlear tissues (see Supplementary data for the complete gene list). The RT<sup>2</sup> Profiler PCR Array (SABiosciences Corp.) is a commercially available set of optimized RT-qPCR primer assays on a 96-well plate that covers different biological pathways (Hu et al., 2009; Joyce et al., 2011).

The array contains 84 primer pairs of oxidative stress and antioxidant defense pathway-focused genes and 5 primers of housekeeping genes: ribosomal protein, large, P1 (Rplp1), hypoxanthine phosphoribosyltransferase 1 (Hprt1), ribosomal protein L13A (Rpl13a), lactate dehydrogenase A (Ldha), and actin, beta (Actb). Seven wells were used to test nontranscribed genomic DNA contamination and PCR performance.

## 2.6. Tissue collection and RNA preparation

One array required purified RNAs from both cochleae in each animal. Four arrays from 4 different animals per group (4 biological replicates) were run and analyzed. Animals were deeply anesthetized with CO<sub>2</sub> and decapitated at least 9 days after functional testing. The cochleae were quickly removed and perfused through the round window with RNAlater (Qiagen, Valencia, CA, USA) to preserve RNAs. The cochleae were carefully dissected in RNAlater to collect and pool the CSE and the LWs from the 2 cochleae of each animal.

Total RNA was extracted using the Qiagen RNeasy Micro Kit after tissue homogenization. RNA quantity and quality were evaluated with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). All samples demonstrated good quality RNA (A260/A280 > 1.8 and RNA integrity number (RIN) > 7.5). RIN (Agilent Technologies) is an index of RNA quality based on features of capillary electrophoretograms, with RIN 0 as the most degraded and 10 as the most intact.

## 2.7. cDNA synthesis and RT-qPCR

Upon completion of total RNA extraction, first-strand cDNA was synthesized using 250 ng total RNA from each sample and oligo-dT primed reverse transcription (RT<sup>2</sup> first strand kit, C-03, SABiosciences Corp.) according to the manufacturer's instructions. cDNA product was used in the RT-qPCR array immediately after synthesis.

Gene arrays were processed according to the manufacturer's instructions. First, RT<sup>2</sup> SYBR Green/fluorescein qPCR Master Mix (PA-011-12, SABiosciences Corp.) was mixed with cDNA product. Then 25  $\mu$ L of the aliquot mixture was loaded onto each well of a 96-well array. Real-time RT-qPCR on the array was performed using the Bio-Rad MyiQ Single-Color Real-Time PCR Detection System (MyiQ Optical Module, Bio-Rad Laboratories, Inc., Hercules, CA, USA). The PCR reaction was run with a 2-step cycling program (1 cycle at 95 °C for 10 minutes for initial denaturation; 40 cycles consisting of 15 seconds at 95 °C for denaturing, and 1 minute at 60 °C for annealing).

Upon completion of the PCR run, threshold cycle (Ct) values were calculated using default conditions of the iQ5™ Optical System Software (Version 2.1, Bio-Rad Laboratories, Inc.). The mean threshold of all genes on all arrays was used to obtain Ct values for all genes. Four biological replications of the real-time RT-qPCR were obtained per group to calculate *p*-values. RT-qPCR efficiency was evaluated by the array-implemented testing and was approximated by the shape of the logarithmic PCR amplification plot (Schmittgen and Livak, 2008), which suggested good efficiency in all arrays.

## 2.8. Statistical analysis

A 2-way analysis of variance (ANOVA) was employed to assess differences in ABR thresholds (group × frequency) and DPOAE amplitudes (group × F2 intensity) using GraphPad Prism 5 software (Version 5.01, GraphPad Software Inc., La Jolla, CA). Frequency and intensity were treated as repeated measures. Bonferroni post hoc analysis was performed to detect which frequencies showed significant differences.

To determine the most appropriate reference gene among 5 housekeeping genes in RT-qPCR, fold change (FC) of a given group relative to others was calculated using the formula of Schmittgen and Livak, 2008:

$$\begin{aligned} FC_{\text{middle (vs.young)}} &= \text{mean } 2^{-Ct_{\text{middle-age group}}} / \text{mean } 2^{-Ct_{\text{young-age group}}}; \\ FC_{\text{old (vs.young)}} &= \text{mean } 2^{-Ct_{\text{old-age group}}} / \text{mean } 2^{-Ct_{\text{young-age group}}}; \\ FC_{\text{old (vs.middle)}} &= \text{mean } 2^{-Ct_{\text{old-age group}}} / \text{mean } 2^{-Ct_{\text{middle-age group}}}; \end{aligned}$$

A Student *t* test was performed on  $2^{-Ct}$ , and the gene which showed the smallest FC and largest *p*-value was selected as a reference gene for RT-qPCR analysis. The average of normalized mRNA levels in individual cochlear genes in the young-age group are presented as mean  $2^{-Ct} \pm$  SD (standard deviation), where  $Ct = Ct_{\text{target gene}} - Ct_{\text{reference gene}}$  (Schmittgen and Livak, 2008). Coefficient of variation (CV) was computed as a percentage of the ratio of the SD to the mean to express the interarray variation.

For gene expression profiling experiments, a comparative Ct method was used to present the data as FC in expression (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008). The online Excel-based RT<sup>2</sup> Profiler PCR Array Data Analysis Template v3.1, which was prepared by SABiosciences Corp., was modified and used to evaluate the age-related cochlear gene expression changes. Specifically, the expression levels of target genes were first normalized to the reference gene to generate the  $Ct$ . Then, the following formulas were used (Schmittgen and Livak, 2008):

$$\begin{aligned} FC_{\text{middle (vs.young)}} &= \text{mean } 2^{-\Delta Ct_{\text{middle-age group}}} / \text{mean } 2^{-\Delta Ct_{\text{young-age group}}}; \\ FC_{\text{old (vs.young)}} &= \text{mean } 2^{-\Delta Ct_{\text{old-age group}}} / \text{mean } 2^{-\Delta Ct_{\text{young-age group}}}; \\ FC_{\text{old (vs.middle)}} &= \text{mean } 2^{-\Delta Ct_{\text{old-age group}}} / \text{mean } 2^{-\Delta Ct_{\text{middle-age group}}}; \end{aligned}$$

When  $FC < 1$ , the negative inverse of the FC ( $-1/FC$ ) was used to express the FC reduction in expression. A statistical analysis of significance in FC was accomplished with the Student *t* test on  $2^{-Ct}$ .

Pearson correlation coefficient ( $r$ ) and  $p$ -values were computed with GraphPad Prism 5 software to investigate the relationship between: (1) gene expression ( $- Ct$ ) and average ABR thresholds (20, 30, and 40 kHz) or average DPOAE amplitudes ( $L_1/L_2 = 65/55$  dB SPL;  $F_2 = 4, 8, 11.3, \text{ and } 16$  kHz), and (2) expression of PCR-significant glutathione- and thioredoxin-related genes.

### 3. Results

#### 3.1. ABR and DPOAE

Fig. 2 shows mean ( $\pm$  SD) ABR thresholds at 5, 10, 15, 20, 30, and 40 kHz in the young ( $n = 10$ ), middle-age ( $n = 8$ ), and old ( $n = 10$ ) F344/NHsd rats. When the ABR thresholds exceed the limit of the testing paradigm (90 dB SPL), the mean ABR threshold is calculated using 90 dB SPL for that datum point. Two-way ANOVA and post hoc tests showed statistically significant differences in ABR thresholds at 20 ( $p < 0.01$ ), 30 ( $p < 0.001$ ), and 40 kHz ( $p < 0.001$ ) in the comparison of the young- and middle-age groups. Young versus old and middle versus old comparisons showed statistically significant group difference at all frequencies ( $p < 0.001$ ). The results indicated a gradual age-related increase in ABR thresholds starting from high frequency regions and expanding to low frequencies. This is consistent with previous studies (Bielefeld et al., 2008; Chen et al., 2009; Hu et al., 2008).

Fig. 3 illustrates mean ( $\pm$  SD) DPOAE input/output (I/O) functions at 4 (A), 8 (B), 11.3 (C), 16 (D), and 20 (E) kHz of  $F_2$  frequencies in the young ( $n = 10$ ), middle ( $n = 8$ ), and old ( $n = 10$ ) age groups. A 2-way ANOVA (frequency  $\times$  age) revealed that DPOAE I/O functions in all groups were significantly different from each other ( $p < 0.05$ ) at all frequencies, except for the comparison of the young- and middle-age groups at 20 kHz. These decreases indicate the presence of age-related OHC dysfunction starting from at least middle age.

DPOAE/OHC dysfunction from 4 to 16 kHz in the middle-age group was not reflected in ABR threshold change. However, the old age group showed a significant change in both ABR and DPOAE compared with the young- and middle-age groups.

Otoscopic examinations revealed cerumen-free external canals in the young-age group, minimal cerumen in the middle-age group, and severe cerumen accumulation deep in the external canal near the tympanic membrane in the old animals. Attempts were made to remove cerumen in some old rats; however, DPOAE amplitude after cleaning either decreased or remained the same. Therefore, data from these animals were not included in the data analysis. Due to the difficulty in cerumen removal and lack of any benefit, the decision was made to not clean the cerumen in the experimental animals to avoid potential damage to the tympanic membrane. It is conceivable that the decrease in DPOAE amplitudes between middle and old age may be due, in part, to the accumulation of cerumen; however, we believe that this is unlikely. First, removing the cerumen failed to increase DPOAE amplitudes. Second, the age-related decrease in DPOAE amplitudes was frequency-dependent, being greater at high than low frequencies; cerumen obstruction of the ear canal would be expected to cause a relatively flat, frequency-independent loss.



### 3.2. Cytocochleogram

Fig. 3F shows the mean ( $n = 3$  per group;  $\pm$  SD) cytocochleogram showing OHC loss in the young-, middle-, and old-age groups. The upper x-axis shows the frequency that corresponds to the cochlear location on the lower x-axis. The young- and middle-age groups showed similar cytocochleograms with little OHC loss, with the possible exception of the middle-age group which had  $< 20\%$  OHC loss in the basal turn ( $> 90\%$  from the apex), which corresponds to frequencies above 40 kHz. In contrast, the old-age group had a U-shaped cytocochleogram (more OHC loss in the apical and basal turns and intact OHC in the middle turn), consistent with previous studies (Bielefeld et al., 2008; Chen et al., 2009; Hu et al., 2008).

### 3.3. RT-qPCR array

**3.3.1. Reference gene selection**—To assess the stability of the reference genes for the 3 age groups used for RT-qPCR analysis, mRNA expression levels of 5 housekeeping genes in the array (Rplp1, Hprt1, Rpl13a, Ldha, and Actb) were compared between different age groups (young vs. middle, young vs. old, and middle vs. old) using the Student *t* test. Table 1 shows *p*-values and FC values for these reference genes. Ldha, the gene that encodes the enzyme that catalyzes the conversion of L-lactate to pyruvate in the final step of anaerobic glycolysis, demonstrated the highest *p*-values, and the FC values were closest to 1.0 for all age group comparisons. Therefore, Ldha was selected as a reference gene for RT-qPCR analysis.

### 3.4. RT-qPCR gene expression profile

The normalized mRNA levels of individual genes in the young age group are presented as mean  $2^{-Ct} \pm$  SD, and the interarray variation is shown as CV, as expressed in percentage (Table 2). In the young group, hemoglobin alpha, adult chain 2 (Hba-a2) and apolipoprotein E (ApoE) were the 2 most highly expressed genes among the 84 genes tested. Gpx4 was also highly expressed in the young cochleae, followed by other isoforms: Gpx3, Gpx1, Gpx2, Gpx6, Gpx8, Gpx7, and Gpx5, from high to low expression. All 3 types of Cu/Zn-superoxide dismutase (Sod) genes showed high expression levels in the order of Sod3, Sod1, and Sod2. The peroxiredoxin (Prdx) family members, Prdx1 and Prdx2, were highly expressed in the young cochleae, followed by Prdx3, Prdx6, Prdx4, and Prdx5.

Table 2 also presents percent CV values in the young group ranging from 4.35% to 37.51%, with a mean percentage of 15.53. This indicates that individual genes exhibit their own interarray variation, which may reflect intersubject differences in expression levels of these genes.

The effects of age on cochlear gene expression are shown in Table 3; comparisons are shown for young versus middle-age; young versus old, and middle-age versus old. Significant age-related changes in cochlear gene expression were observed in 13 genes. One gene, stearoyl-coenzyme A desaturase 1 (Scd1), showed significant downregulation at 21 months of age relative to 2- and 12-month-old rats ( $-1.18$  FC, with  $p < 0.05$ , and  $-1.25$  FC, with  $p < 0.01$ , respectively); the gene expression in young- and middle-age groups was similar.

Twelve genes, glutathione peroxidase 6 (Gpx6), glutathione *S*-transferase, kappa 1 (Gstk1), thioredoxin interacting protein (Txnip), glutathione peroxidase 3 (Gpx3), Gsr, cytoglobin (Cygb), dual oxidase 2 (Duox2), aminoadipatesemialdehyde synthase (Aass), solute carrier Family 38, Member 5 (Slc38a5), NAD(P)H dehydrogenase, quinone 1 (Nqo1), vimentin (Vim), and 24-dehydrocholesterol reductase (Dhcr24), were upregulated in the old age group, with the mean FC ranging from 1.20 to 1.71. Interestingly, 5 upregulated genes (Gpx6, Gstk1, Txnip, Gpx3, and Gsr) related to glutathione metabolism and thioredoxin regulation showed statistically significant upregulation in the middle- age group. Gpx6, Gstk1, and Txnip were upregulated in middle- and old-age groups relative to young-age group, but Gsr and Gpx3 mRNA levels were upregulated only in middle- age group relative to young-age group. Six genes (Cygb, Duox2, Aass, Slc38a5, Nqo1, and Vim) were upregulated only when the animals reached old age, and Dhcr24 was significantly upregulated in the old age group compared with the middle-age group.

### 3.5. Correlation between gene expression and hearing measurements

To examine the relationship between gene expression and hearing measurements, linear correlation analyses were performed on 13 PCR-significant genes. Table 4 shows Pearson correlation coefficient (*r*) and *p*-values between gene expression (– Ct) and average ABR thresholds (20, 30, and 40 kHz) or average DPOAE amplitudes ( $L_1/L_2 = 65/55$  dB SPL,  $F_2 = 4, 8, 11.3,$  and 16 kHz).

Significant linear correlations were found in 8 genes (Scd1, Cygb, Duox2, Aass, Slc38a5, Nqo1, Vim, and Dhcr24), with either ABR/DPOAE or both. Glutathione and thioredoxin-related PCR-significant gene (Gpx6, Gstk1, Txnip, Gpx3, and Gsr) expressions were not significantly correlated with either ABR thresholds or DPOAE amplitudes, and these are the 5 genes that showed significant upregulation in the middle-age group. Cygb demonstrated the highest *r* value and lowest *p*-values, indicating the strongest correlations with ABR and DPOAE.

### 3.6. Correlation between gene expression of PCR-significant genes related to glutathione and thioredoxin

To examine whether the expression changes in glutathione- and thioredoxin-related genes (Gpx3, Gpx6, Gsr, Gstk1, and Txnip) are correlated with each other, Pearson correlation coefficients (*r*) and *p*-values were obtained (Table 5). The analyses revealed significant correlations between Gpx3, Gpx6, Gsr, and Gstk. Expression of Txnip was correlated with all glutathione-related gene expression, except for Gstk1.

## 4. Discussion

### 4.1. The effects of age on hearing and OHC loss

The current results from ABR and DPOAE (Figs. 2 and 3) are consistent with previous results (Bielefeld et al., 2008; Chen et al., 2009; Hu et al., 2008) that show a decrease in auditory function with aging and that confirm a discrepancy between reduced DPOAE amplitudes and the intact OHCs in the middle frequency regions in the old-age group. The cytochleograms in the middle- and old-age groups showed no significant OHC loss across

the 5–40- kHz region of the cochlea, but the DPOAE amplitudes were significantly decreased in this region. Therefore, the agedependent decreases in DPOAE amplitude are not likely due to OHC loss but to other possible factors, including decreased expression of prestin in OHC (Chen et al., 2009); impairment in the stria vascularis and endolymphatic potential; tectorial membrane distortion (Buckiova et al., 2006); or functional impairment in inner hair cells/nerve fibers/spiral ganglion cells (Chen et al., 2009).

## 4.2. Genomic analysis

**4.2.1. Cochlear gene profile**—The 5 cochlear genes that showed highest mRNA expression levels in young F344/NHsd rats were Hba-a2, Apoe, Gpx4, Prdx1, and Gpx3. The gene that showed the highest mRNA expression, Hba-a2, is an oxygen transporter and a member of the globin family. The second most abundant gene, Apoe, is essential for the normal catabolism of triglyceride-rich lipoprotein constituents. Apoe is the single most important risk factor for Alzheimer's disease (Bertram and Tanzi, 2010). The third and fifth most abundant genes, Gpx4 and Gpx3, members of the glutathione peroxidase family, are a part of the glutathione antioxidant system. Both isoforms catalyze the reduction of hydrogen peroxide or organic peroxides to water or to corresponding alcohols using reduced glutathione as a specific electron donor substrate (Margis et al., 2008). Gpx4 (phospholipid Gpx) is bound to membranes and distributed in the nucleus, cytosol, and mitochondria. Gpx4 has the unique ability to reduce fatty acid and cholesterol hydroperoxides to alcohol by acting upon peroxidized fatty acid residues within membranes and lipoproteins (Halliwell and Gutteridge, 2007). Gpx3 (plasma Gpx) is a glycoprotein whose role remains poorly understood (Brigelius-Flohé, 2006), although it may protect the kidney from oxidative damage. The fourth most abundant mRNA, Prdx1, is a cytosolic peroxiredoxin that encodes antioxidant enzymes that reduce hydrogen peroxide and alkyhydroperoxides dependent on thioredoxin (Watanabe et al., 2010). Interestingly, Prdx2, the only peroxiredoxin isoform tested in the Hu et al. (2009) study, was 1 of the 3 most highly expressed genes in young Sprague-Dawley rat cochlea. In addition, Kong et al. (2009) reported decreases in thioredoxin and thioredoxin reductase gene expression and protein levels in *tubby* mice (*tub/tub*) compared with heterozygous (*tub/wt*) or wild type (*wt/wt*). The *tubby* phenotype expresses late-onset weight gain and progressive loss of hair cells and retinal degeneration (Ohlemiller et al., 1995, 1997).

**4.2.2. Effects of age on gene expression**—In our PCR array experiments, 1 gene (*Scd1*) was downregulated, while 12 genes (*Gpx6*, *Gpx3*, *Gstk1*, *Txnip*, *Gsr*, *Cygb*, *Duox2*, *Aass*, *Slc38a5*, *Nqo1*, *Vim*, and *Dhcr24*) were upregulated due to aging. Based on the age-related degeneration (i.e., OHC loss) in the cochlea, the interpretation of the significantly downregulated and upregulated genes needs to be done with caution due to the loss of specific cell types that contribute to the pool of mRNA.

In the current study, the presence of oxidative stress in rats as young as 12 months of age is suggested by upregulation of the genes related to glutathione metabolism (*Gpx6*, *Gpx3*, *Gstk1*, and *Gsr*). This early upregulation of oxidative stress genes could account for the early onset of oxidative stress and increases in mitochondrial DNA deletions in the F344/NHsd's cochlea at 6 months of age that were reported by the Yin et al. (2007) study.

Likewise, the significant upregulation of Gpx genes is consistent with upregulation of Gpx protein activity in the F344/NHsd rats (Coling et al., 2009).

Significant linear correlations among Gpx6, Gpx3, Gstk1, and Gsr gene expressions suggest that these genes are coregulated by a common factor, probably by an antioxidant response element (ARE) regulatory region in the promoter region. These genes presumably contain ARE, which is a cis-acting enhancer sequence in the transcriptional regulatory regions (Nioi and Hayes, 2004). AREs have cytoprotective functions. In response to oxidative stress, nuclear factor-erythroid 2 p45-related Factor 2 (Nrf2) binds to ARE and induces expression of a cluster of genes that encode antioxidant and xenobiotic-metabolizing enzymes (Halliwell and Gutteridge, 2007; Kensler et al., 2007; Nioi and Hayes, 2004). The upregulation of the ARE-inducible genes in the current study strongly suggests that the cochlear tissue is under oxidative stress. Given these results, it would be interesting to study the role of the kelchlike ECH-associated protein 1 (Keap 1)-Nrf2-ARE pathway in ARHL. Txnip, originally identified as vitamin D3 upregulated Protein 1 (Vdup1), also showed significant upregulation (Chen and DeLuca, 1994).

Txnip is thought to negatively regulate thioredoxin (Nishiyama et al., 1999), but recent evidence suggests that Txnip may be involved in lipid/glucose metabolism (Watanabe et al., 2010) because there is an inverse correlation between its gene expression and longevity (Chondrogianni et al., 2004; de Candia et al., 2008; Yoshida et al., 2006). The significant upregulation of Txnip in the middle- and old-age groups may imply a depletion of cochlear thioredoxin and changes in cochlear lipid/glucose metabolism.

Txnip could conceivably be used as a target to increase cochlear thioredoxin levels to prevent ARHL. Kong et al. (2009) demonstrated that sulforaphane, a naturally occurring isothiocyanate, protected against *tub*-related cochlear degeneration thought to be caused by downregulation of the thioredoxin antioxidant system. Thioredoxin has not been extensively studied as a treatment for acquired hearing loss (noise-induced, drug-induced, and age-related hearing loss). Upregulation of the thioredoxin defense system may be an important therapeutic option for hearing protection.

Cygb is the gene that had the highest FC and the lowest *p*-values in the old-age group relative to the young-age group. Cygb is a member of a newly-discovered class of the hexacoordinate hemoglobin superfamily. Globins are oxygen-binding molecules, with 5 types identified in vertebrates: hemoglobin, myoglobin, neuroglobin, cytoglobin, and globin X. Among 4 genes tested (hemoglobin, myoglobin, neuroglobin, and cytoglobin), our study detected only Cygb and hemoglobin mRNA in the F344/NHsd cochlea.

Cygb showed the strongest linear correlation with ABR/DPOAE, indicating that Cygb may play an important role in ARHL. In mammals, Cygb mRNA is present at higher concentrations in cells associated with high metabolic rates such as those in the brain, eyes, liver, heart, and skeletal muscle (Burmester et al., 2002; Fordel et al., 2004). It is notable that significant age-related upregulation of Cygb gene expression was reported in human corneal endothelium in old subjects ( $\geq 50$ -year-old) compared with young ( $\geq 30$ -year-old; Joyce et al., 2011).

Cygb may be a promising target for ARHL prevention because it works not only as a ROS scavenger but also as a generator of an antioxidant response. Initially, Cygb was suggested to play an important role in fibrogenesis in the liver and in the protection of cells from oxidative stress by functioning as a ROS scavenger (Fordel et al., 2007; Li et al., 2007). New evidence indicates that Cygb interacts with and oxidizes lipids under an oxidative environment, generating cell-signaling lipid molecules, inducing cell-signaling pathways, and creating an antioxidant response to the oxidative stress (Reeder et al., 2011).

**4.2.3. Relation between gene expression and hearing measurements**—Linear correlation analyses were used to investigate whether there were significant relationships between gene expression and auditory function, similar to what was done with large-scale gene arrays in the CBA mouse cochlea (Tadros et al., 2008). Most of the significant gene expression changes were strongly correlated with hearing measurements. This suggests that these genes may contribute significantly to ARHL. However, this interpretation is not simple or straightforward because many of the gene expression changes associated with ARHL have cytoprotective functions, or have both survival and apoptotic functions. The upregulation of cytoprotective genes could be a natural response to counteract oxidative stress that leads to ARHL. At some point, however, the survival-promoting aspects of some genes may switch to apoptosis when cell survival is no longer a viable option.

Interestingly, the expression changes of the glutathione-related genes were not linearly related to the deterioration in ABR and DPOAE. This occurred because the expression levels of glutathione-related genes were upregulated early and did not increase further with advancing age, whereas auditory function progressively deteriorated with advancing age. These results suggest that the glutathione antioxidant system is the first defense that is upregulated to suppress oxidative stress and minimize ARHL and tissue damage. However, because ARHL continues to accumulate, the upregulation of glutathione genes is not sufficient to fully suppress oxidative stress, leading to further progression of ARHL. In other words, upregulation of glutathione genes is not sufficient to completely protect the cochlea in ARHL. Because the mean FCs measured in these glutathione-related genes were relatively low, supplementing of exogenous glutathione-related antioxidant agents could be a reasonable option for ARHL treatment.

#### 4.3. Future studies with rat ARHL models

The current study was designed to serve as the first phase to identify significant cochlear genes in the oxidative stress and antioxidant defense pathway in relation to ARHL in the F344/NHsd rat. Our results identified specific subsets of oxidative stress genes that appear to play an important role in ARHL in the F344/NHsd rats. The RT-qPCR array experiments revealed significant downregulation in 1 gene (*Scd1*) and upregulation in 12 genes (*Gpx6*, *Gpx3*, *Gstk1*, *Txnip*, *Gsr*, *Cygb*, *Duox2*, *Aass*, *Slc38a5*, *Nqo1*, *Vim*, and *Dhcr24*) due to aging. Among these genes, *Txnip* and *Cygb* as well as glutathione-related genes seem to be promising targets for ARHL prevention.

Because the current gene expression studies were conducted with tissues from the whole cochlea, significant gene expression changes limited to a particular region of the cochlea or

specific cell type would likely have been overlooked in the current study. However, because our gene profiling detected age-related gene expression changes in the whole cochlear mRNA, the changes we observed must be occurring at high levels in specific regions or cell types, or at moderate levels across most cochlear tissues. Regardless of whether the changes are region-specific or global in nature, the observed changes provide novel mechanistic insights and potential opportunities for therapeutic intervention.

There is an inborn weakness of all gene array studies, which is that differences in mRNA levels do not necessarily translate to changes in protein levels. Therefore, future investigations of protein gene expression changes in specific cochlear cell types or tissue compartments are imperative, and immunostaining will offer the possibility of identifying significant regional changes that are likely to be essential for achieving a full picture of oxidative stress in the F344/NHsd rat.

ARHL is a complex sensory disorder caused by the interaction of a variety of factors, including genetic and nongenetic factors (Van Eyken et al., 2007). Unlike our current lack of knowledge of the rat ARHL model, the genetic causes of ARHL are well understood in mouse ARHL models. This understanding makes it easier to investigate whether oxidative stress is an initial cause or a downstream event in ARHL. For example, CBA/J mice carry the age-related hearing loss (Ahl)-resistant gene (Johnson et al., 1997) and develop ARHL much later in life compared with C57BL/6J and DBA/2J, which show cadherin23 deficits in stereocilia and the involvement of the Ahl1 locus (Johnson et al., 2008; Nemoto et al., 2004; Noben-Trauth and Johnson, 2009). Age-related increase in immunoreactivity of selected oxidative stress-related markers (glutathione-conjugated proteins, anti-4-hydroxynonenal, anti-3-nitrotyrosine) was reported in CBA/J mice (Jiang et al., 2007), indicating that oxidative stress was an initial cause of cochlear tissue degeneration, unlike in C57BL/6J and DBA/2J mice.

The genetic contributions to ARHL in the F344/NHsd have not been fully investigated at this point. It is possible that certain gene alleles in the F344/NHsd initially cause ARHL, and oxidative stress may be a downstream event. Until we have a fuller understanding of the genetic contributions to ARHL in the F344/NHsd, it is difficult to designate an oxidative stress or antioxidant-related gene as a causative “presbycusis gene” in this model.

Nevertheless, we can engineer primary defects in antioxidant genes using a knockout (KO) model to study the effects of these genes on ARHL. Studies of ARHL in KO mouse models suggest that the effects of targeted deletions of particular antioxidant genes, such as Gpx1 and Sod1, may be confounded by genetic backgrounds (Johnson et al., 2010; Keithley et al., 2005; McFadden et al., 1999a, 1999b; Ohlemiller et al., 1999, 2000), and this can mimic the expected interplay of ARHL-inducing genes and antioxidant defense genes in humans. Recently, a knockout rat became available and Knockout Rat Consortium is an excellent website for cutting-edge technologies (<http://www.knockoutrats.org>). The F344 rat and other rat strains, such as Long Evans rats, may offer alternative models or a complement to mice by providing different genetic backgrounds that contribute to ARHL. To realize such research, a more complete understanding of rat ARHL model genomes is critical.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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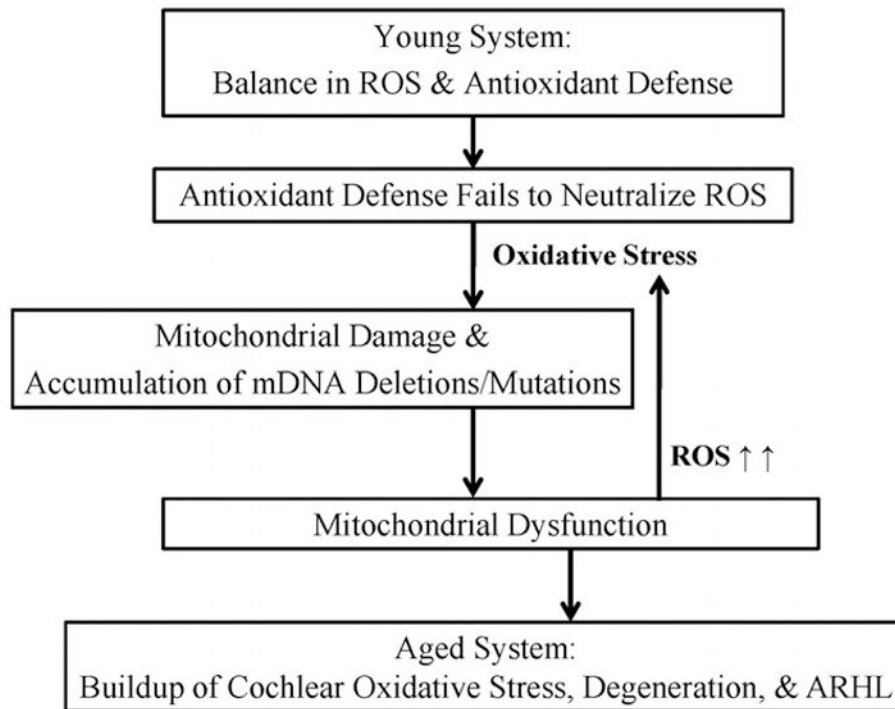
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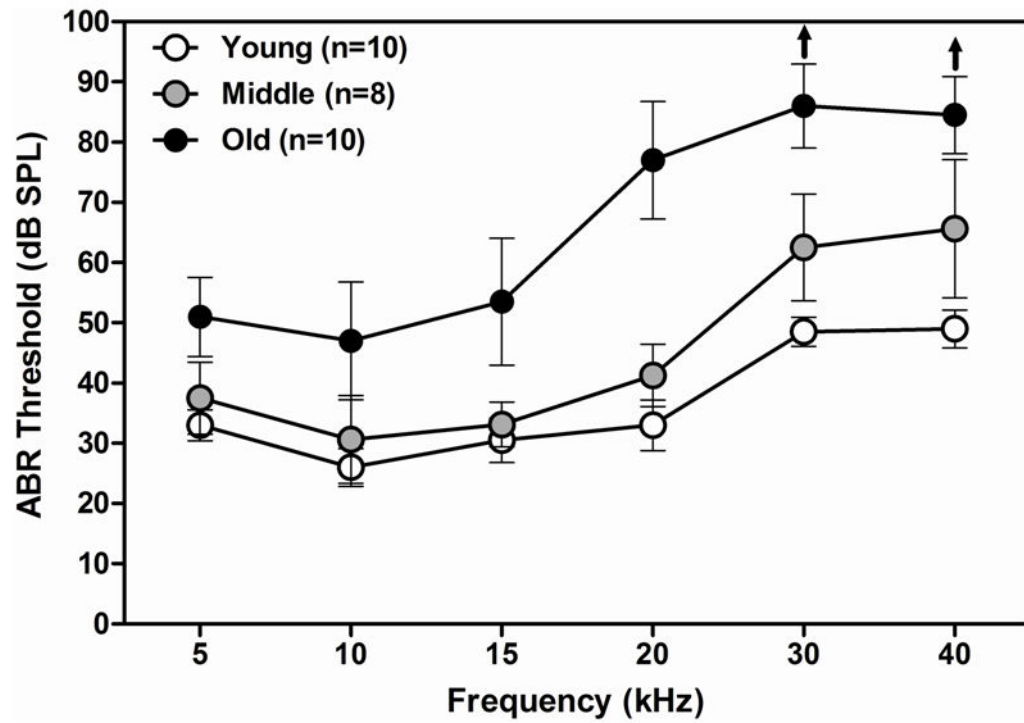


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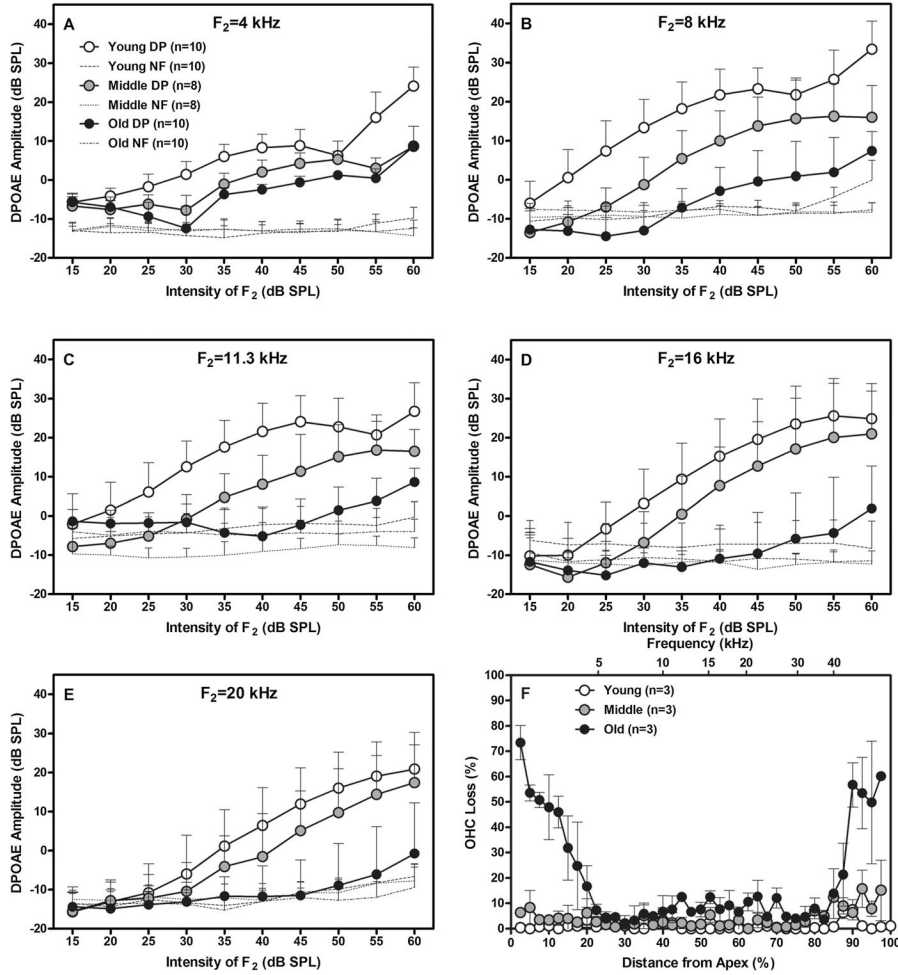
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**Fig. 1.** Oxidative stress and age-related hearing loss (ARHL). Key: mDNA, mitochondrial DNA, ROS, reactive oxygen species.



**Fig. 2.** Mean ( $\pm$  SD) auditory brainstem response (ABR) thresholds at 5, 10, 15, 20, 30, and 40 kHz in the young (open circles), middle-aged (gray circles), and old (filled circles) F344/NHsd. \*  $p > 0.05$ . \*\*  $p < 0.01$ . \*\*\*  $p < 0.001$ .



**Fig. 3.** Mean ( $\pm$  SD) distortion product otoacoustic emission (DPOAE) input/output (I/O) functions at different F<sub>2</sub> frequencies in young-, middle-, and old age groups (A–E); Mean ( $\pm$  SD) percentage of outer hair cell (OHC) loss as a function of distance from the apex (%) in young-, middle-, and old-age groups (F). The upper x-axis shows the frequency that corresponds to the cochlear location on the lower x-axis.

**Table 1**

*p*-values and fold change (FC) for candidate reference genes.

Official Symbol	Middle vs. young	Old vs. young	Old vs. middle
Rplp1	0.137 (1.26)	0.110 (1.23)	0.828 (-1.03)
Hprt1	0.084 (1.24)	0.404 (1.11)	0.278 (-1.12)
Rpl13a	0.042 (1.37)	0.080 (1.24)	0.318 (-1.10)
Ldha	0.938 (1.02)	0.846 (1.03)	0.940 (1.02)
Actb	0.189 (1.24)	0.275 (1.15)	0.654 (-1.07)

**Table 2**

Normalized mRNA levels of individual gene in the young-age group expressed as mean  $2^{-Ct} \pm SD$ , and the interarray variation shown as coefficient of variation (CV) as a percentage

Official Symbol	Mean $2^{-Ct} \pm SD$	%CV
Hba-a2	13.236 $\pm$ 3.134	23.68
ApoE	13.029 $\pm$ 1.063	8.16
Gpx4	5.671 $\pm$ 0.264	4.66
Prdx1	4.203 $\pm$ 0.207	4.94
Gpx3	4.039 $\pm$ 0.369	9.14
Sod3	3.709 $\pm$ 0.384	10.35
Prdx2	3.632 $\pm$ 0.765	21.08
Ctsb	3.581 $\pm$ 0.837	23.37
Scd1	3.537 $\pm$ 0.237	6.69
Sod1	2.586 $\pm$ 0.359	13.87
Tmod1	2.433 $\pm$ 0.169	6.93
Sod2	2.189 $\pm$ 0.223	10.19
Gpx1	1.939 $\pm$ 0.184	9.48
PsmB5	1.854 $\pm$ 0.353	19.06
Park7	1.793 $\pm$ 0.266	14.81
Slc41a3	1.088 $\pm$ 0.154	14.20
Gpx2	1.027 $\pm$ 0.087	8.47
Gpx6	0.796 $\pm$ 0.067	8.45
Prdx3	0.777 $\pm$ 0.063	8.08
Vim	0.771 $\pm$ 0.119	15.46
Pmp	0.705 $\pm$ 0.100	14.18
Txnip	0.625 $\pm$ 0.082	13.11
Aass	0.615 $\pm$ 0.035	5.72
Slc38a1	0.601 $\pm$ 0.122	20.23
Prdx6	0.578 $\pm$ 0.051	8.85
Ppp1r15b	0.536 $\pm$ 0.078	14.55
Ccs	0.502 $\pm$ 0.033	6.51
Gstk1	0.466 $\pm$ 0.033	7.19
Idh1	0.452 $\pm$ 0.058	12.84
Gpx8	0.450 $\pm$ 0.027	6.01
Gsr	0.434 $\pm$ 0.048	11.12
Prdx4	0.405 $\pm$ 0.046	11.26
Cat	0.371 $\pm$ 0.016	4.35
Apc	0.364 $\pm$ 0.039	10.61
Gab1	0.358 $\pm$ 0.066	18.45

Official Symbol	Mean 2 <sup>-</sup> Ct ± SD	%CV
Nxn	0.354 ± 0.051	14.47
Prdx5	0.306 ± 0.058	18.81
Ift172	0.185 ± 0.037	19.80
Ercc2	0.167 ± 0.028	16.57
Xpa	0.167 ± 0.032	19.19
Aqr	0.164 ± 0.015	9.37
Als2	0.113 ± 0.023	20.10
Txnrd2	0.103 ± 0.009	8.93
Ehd2	0.097 ± 0.020	20.83
Ercc6	0.092 ± 0.016	17.26
Gpx7	0.076 ± 0.008	11.13
Dnm2	0.075 ± 0.011	15.31
Txnrd1	0.069 ± 0.011	16.40
Ptgs2	0.055 ± 0.015	27.33
Fancc	0.049 ± 0.009	17.82
Nqo1	0.040 ± 0.007	18.34
Ncf1	0.040 ± 0.008	20.42
Dhcr24	0.037 ± 0.009	23.49
Nudt1	0.033 ± 0.006	17.50
Slc38a5	0.030 ± 0.006	19.13
Cygb	0.023 ± 0.003	12.93
Nudt15	0.019 ± 0.004	20.98
Ncf2	0.014 ± 0.003	23.18
Noxa1	0.011 ± 0.002	20.61
Ucp3	0.010 ± 0.001	7.56
Ptgs1	0.010 ± 0.003	29.16
Kif9	0.009 ± 0.002	18.72
Epx	0.008 ± 0.003	33.35
Fmo2	0.008 ± 0.001	16.53
RGD1560658	0.007 ± 0.002	24.63
Nos2	0.007 ± 0.002	37.51
Slc38a4	0.005 ± 0.001	17.85
Duox2	0.004 ± 0.001	22.19
Zmynd17	0.004 ± 0.0004	9.56
Gpx5	0.004 ± 0.001	29.57
Duox1	0.002 ± 0.0003	20.14



Table 3

FC and *p*-values for 13 PCR-significant genes

Gene Name	Official Symbol	Description	Middle (re: young)		Old (re: young)		Old (re: middle)	
			FC	P-value	FC	P-value	FC	P-value
<b>Downregulated gene</b>								
Stearyl-Coenzyme A desaturase 1	Scd1	Enzyme involved in the synthesis and regulation of unsaturated fatty acids	1.06	0.272	-1.18	0.024*	-1.25	0.007**
<b>Upregulated genes</b>								
<i>Upregulated in middle and old age relative to young age</i>								
Glutathione peroxidase 6	Gpx6	Enzyme to detoxificate hydrogen peroxide	1.44	0.003**	1.30	0.004**	-1.11	0.203
Glutathione S-transferase kappa 1	Gstk1	Function in cellular detoxification	1.29	0.026*	1.20	0.007**	-1.08	0.385
Thioredoxin interacting protein	Txnip	Negatively regulates cytoprotective thioredoxin	1.29	0.018*	1.22	0.027*	-1.06	0.380
<i>Upregulated only in middle age relative to young age</i>								
Glutathione peroxidase 3	Gpx3	Plasma glutathione which detoxificate hydrogen peroxide	1.36	0.011*	1.32	0.089	-1.03	0.792
Glutathione reductase	Gsr	Catalyzes the conversion of GSH to GSSG	1.23	0.029*	1.11	0.148	-1.11	0.117
<i>Upregulated in old age relative to young age</i>								
Cytoglobin	Cygb	Hexacoordinate hemoglobin that may scavenge nitric oxide or other ROS	1.08	0.294	1.71	0.016*	1.58	0.022*
Dual oxidase 2	Duox2	A member of the NADPH oxidase family which catalyze the generation of ROS	1.40	0.087	1.59	0.005**	1.13	0.347
Aminoacidpate-semialdehyde synthase	Aass	A bifunctional enzyme in the mammalian lysine degradation pathway	1.15	0.135	1.27	0.0004***	1.11	0.186
Solute carrier family 38, member 5	Slc38a5	System N amino acid transporter which mediate transport of neutral amino acids	1.21	0.089	1.32	0.034*	1.09	0.214
NAD(P)H dehydrogenase, quinone 1	Nqo1	Enzyme that plays an important role in cytoprotection against toxicity of quinones	1.13	0.220	1.32	0.038*	1.16	0.063
Vimentin	Vim	Class III intermediate filament of the cytoskeleton	1.17	0.099	1.32	0.034*	1.13	0.158
<i>Upregulated only in old age relative to middle age</i>								
24-dehydrocholesterol reductase	Dhcr24	Oxidoreductase which catalyzes the reduction of sterol intermediates during cholesterol biosynthesis	1.03	0.835	1.23	0.113	1.20	0.026*

Key: FC, fold change; GSH, glutathione; GSSG, oxidized glutathione; NADPH, nicotinamide adenine dinucleotide phosphate; PCR, polymerase chain reaction; ROS, reactive oxygen species.

\* *p*<0.05,\*\*\* *p*<0.01,

1000<math>p</math>  
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**Table 4**

Pearson correlation coefficients (*r*) and *p*-values between gene expression of PCR-significant genes and hearing measurements

Official symbol	Average ABR, 20, 30, 40 kHz ( <i>r</i> , <i>p</i> )	Average DPOAE, 4, 8, 11.3, 16 kHz ( <i>r</i> , <i>p</i> )
Gpx6	0.414, 0.181	-0.353, 0.261
Gstk1	0.359, 0.252	-0.313, 0.322
Txnip	0.432, 0.161	-0.437, 0.156
Gpx3	0.470, 0.145	-0.301, 0.368
Gsr	0.172, 0.593	-0.256, 0.422
Cygb	0.937, <0.0001 <sup>***</sup>	-0.914, <0.0001 <sup>***</sup>
Duox2	0.651, 0.022 <sup>*</sup>	-0.723, 0.008 <sup>**</sup>
Aass	0.747, 0.008 <sup>**</sup>	-0.709, 0.015 <sup>*</sup>
Slc38a5	0.621, 0.031 <sup>*</sup>	-0.743, 0.006 <sup>**</sup>
Nqo1	0.700, 0.011 <sup>*</sup>	-0.773, 0.003 <sup>**</sup>
Vim	0.750, 0.005 <sup>**</sup>	-0.747, 0.005 <sup>**</sup>
Dhcr24	0.522, 0.082	-0.623, 0.031 <sup>*</sup>
Scd1	-0.703, 0.011 <sup>*</sup>	0.630, 0.028 <sup>*</sup>

Average ABR thresholds at 20, 30, and 40 kHz or average DPOAE amplitudes at L<sub>1</sub>/L<sub>2</sub> = 65/55 dB SPL and F<sub>2</sub> = 4, 8, 11.3, and 16 kHz)

Key: ABR, auditory brainstem response; DPOAE, distortion product otoacoustic emission; PCR, polymerase chain reaction, SPL, sound pressure level.

\* *p* < 0.05;

\*\* *p* < 0.01;

\*\*\* *p* < 0.001.

**Table 5**

Pearson Correlation Coefficients (*r*) and *p*-values in linear correlation analysis in significantly changed glutathione- and thioredoxin-related genes.

	<b>Gpx3</b>	<b>Gpx6</b>	<b>Gsr</b>	<b>Gstkl</b>	<b>Txnip</b>
Gpx3	<i>r</i> N/A	0.797	0.706	0.719	0.794
	<i>p</i> N/A	0.003**	0.015*	0.013*	0.004**
Gpx6	<i>r</i> —	N/A	0.809	0.905	0.700
	<i>p</i> N/A	N/A	0.001**	0.0001***	0.011*
Gsr	<i>r</i>	N/A	N/A	0.735	0.739
	<i>p</i>	N/A	N/A	0.006**	0.006**
Gstkl	<i>r</i>	N/A	N/A	N/A	0.555
	<i>p</i>	N/A	N/A	N/A	0.061
Txnip	<i>r</i>	N/A	N/A	N/A	N/A
	<i>p</i>	N/A	N/A	N/A	N/A

Key: NA, not applicable.

\*  $p < 0.05$ .

\*\*  $p < 0.01$ .

\*\*\*  $p < 0.001$ .