

EFFECT OF SOD1 OVEREXPRESSION ON AGE- AND NOISE-RELATED HEARING LOSS

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Abstract—Reactive oxygen species (ROS) have been implicated in hearing loss associated with aging and noise exposure. Superoxide dismutases (SODs) form a first line of defense against damage mediated by the superoxide anion, the most common ROS. Absence of Cu/Zn SOD (SOD1) has been shown to potentiate hearing loss related to noise exposure and age. Conversely, overexpression of SOD1 may be hypothesized to afford a protection from age- and noise-related hearing loss. This hypothesis may be tested using a transgenic mouse model carrying the human SOD1 gene. Contrary to expectations, here, we report that no protection against age-related hearing loss was observed in mice up to 7 months of age or from noise-induced hearing loss when 8 week old mice were exposed to broadband noise (4–45 kHz, 110 dB for 1 h). Mitochondrial DNA deletion, an index of aging, was elevated in the acoustic nerve of transgenic mice compared to nontransgenic littermates. The results indicate the complexity of oxidative metabolism in the cochlea is greater than previously hypothesized. © 2003 Elsevier Science Inc.

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INTRODUCTION

Sensorineural hearing loss resulting from presbycusis, the normal aging process, is common and contributes to significant disability. The exact mechanism of presbycusis is not established, but is thought to be due to a series of insults to the inner ear over time. These include age-related degeneration, noise exposure, and ear diseases [1]. While the general mechanisms of aging in other tissues also remain to be elucidated, there is strong evidence that increased generation of reactive oxygen species (free radicals) during cellular metabolism plays a major role. In particular, involvement of the superoxide radical ($O_2^{\bullet-}$) in the production of cellular damage in human disease is well supported [2]. Oxidative stress has

also been implicated in presbycusis by reports of increased susceptibility of SOD1 knockout mice [3,4] and increases of SOD1 mRNA in older mice [5].

Excessive noise exposure can also cause hearing loss. Anatomical studies demonstrate tearing of the tectorial membrane and detachment of the organ of Corti from the basilar membrane in response to intense noise and general metabolic degradation of sensory cells in response to sustained high-level noise [6–8]. Despite extensive research, the exact mechanism of damage for these anatomical changes has not been elucidated. One constant finding is altered cochlear microcirculation. Vasoconstriction, increased vascular permeability, aggregations of red blood cells, and edema all contribute to decreased microcirculation and resultant ischemia. Free oxygen radicals are generated in response to prolonged hypoxia or upon reperfusion after ischemic episodes [9–12]. Increased levels of reactive oxygen species have been directly linked to sensory damage and threshold shifts associated with noise exposure [13–17]. In addition,

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Seidman *et al.* demonstrated that intramuscular treatment of rats with SOD-polyethylene glycol before and during noise exposure preserves cochlear sensitivity and postulated that the activity of free oxygen radicals may play a role in noise-induced damage to the cochlea [9,18].

A family of SODs forms the first line of defense against $O_2^{\cdot-}$ mediated damage in the cochlea [3]. SODs catalyze the conversion of $O_2^{\cdot-}$ to hydrogen peroxide and water, thereby preventing direct damage by $O_2^{\cdot-}$. The removal of $O_2^{\cdot-}$ also eliminates its potential conversion to peroxynitrite, a highly toxic metabolite [19]. Of the three known isoforms of SOD, SOD1 is the most abundant in the cochlea, comprising approximately 74% of total SOD activity [20]. Deficiency of SOD1 has been demonstrated to increase the vulnerability of the cochlea to damage associated with normal aging and noise, presumably through metabolic pathways involving $O_2^{\cdot-}$ [3,4]. Here, we investigate whether overexpression of SOD1 in transgenic mice [21] can have a converse effect, *i.e.*, protection from hearing loss associated with aging and noise exposure.

MATERIALS AND METHODS

Animals

C57BL/6-TgN<SOD1>3Cje mice were produced using the human SOD1 gene [21]. They were maintained by backcrossing to C57BL/6J over more than 20 generations. Transgenic animals are hemizygous carrying 7–8 copies of human SOD1 on chromosome 3 [22]. To determine phenotype, 5–10 μ l of blood was collected from tails in 20 μ l lysis buffer containing 0.5% Nonidet P-40 and 1 mM EDTA, pH 8.0, and a broad spectrum of protease inhibitors (Roche, Indianapolis, IN, USA). Proteins were separated by nondenaturing polyacrylamide gel electrophoresis and SOD activity was detected by nitroblue tetrazolium stain as described [21]. Transgenic and nontransgenic littermate control mice were distinguished by the presence of the human SOD1 transgene product. All animals were kept in clean cages located in quiet animal facilities approved and maintained by the Laboratory Animal Resources Center of the University of California, San Francisco. Food and water were available at all times. The experimental protocol was approved by the University of California San Francisco Committee on Animal Research.

Noise exposure

Mice were sedated with an intraperitoneal injection of 100 mg/kg ketamine, 10 mg/kg xylazine and exposed for 1 h to noise from a Radio Shack Super Tweeter model 40–1310B (Tandy Corp., Fort Worth, TX, USA). The acoustic signal was white noise from 4–45 kHz. Noise level was adjusted to 110 dB sound pressure level (SPL)

with a Bruel and Kjaer microphone type 4191 and conditioning amplifier, type 2690 (Naerum, DK).

Measurement of hearing

ABR testing was conducted in a double-walled acoustic chamber (Industrial Acoustics Co., Inc., Bronx, NY, USA). Mice were sedated with ketamine and xylazine as described above. Core temperature was maintained at 36–38°C using a Baxter K module K-20 isotherm heating pad. Silver electrodes were inserted subcutaneously at the vertex (reference A), and just inferior to each ear (ipsilateral, B and contralateral, ground). The biological signal, A-B, referenced to the contralateral ear was amplified using a DAM-50E amplifier (World Precision Instruments, Sarasota, FL, USA), bandpass filtered from 0.3–3 kHz, 90 dB/octave, (Stewart Electronics VBF-8.04, Wayland, MA, USA), digitized (25 kHz sampling rate) and recorded over a 10 ms time window on a Tucker Davis Technologies System II (Gainesville, FL, USA). Stimuli were generated with Tucker-Davis hardware and software in conjunction with a Pentium II computer. Click stimuli were 100 μ s pulses of alternating polarity. Stimuli were presented at a rate of 20/s, amplified by a Samson Servo 170 (Syosset, NY, USA) and delivered by a Radio Shack Super Tweeter modified with an inverted acoustic horn to fit the external ear canal. Peak sound pressure levels were calibrated with a Bruel and Kjaer Sound Level Meter type 2209 fitted with a type 4191 measuring microphone (Naerum, DK). Stimulus level was decreased in 5 dB steps, from 80–0 dB peak sound pressure level. Threshold was determined as the level one half step above the level where no discernible ABR waveform could be discerned. All data were randomly reviewed by the tester several months after recording and compared to the original measurement. Seventy-six percent were within 5 dB of the initial determination, with 100% within 10 dB.

Enzyme assays

Mice were anesthetized and killed by decapitation. 30 μ l blood was collected in 120 μ l lysis buffer (described above). Brain and the bony inner ear, trimmed from the temporal bone were homogenized in lysis buffer at approximately 4 mg/ml total protein and frozen at -80°C . Protein concentration was determined using a bicinchoninic acid assay (Pierce, Rockford, IL, USA). SOD1 activity was detected by nitroblue tetrazolium stain as described [21].

Mitochondrial DNA deletions

Brain, liver, auditory nerve, and lateral wall from 10 transgenic and 10 nontransgenic littermate control mice were stored at -70°C until used. Lateral wall tissue consisted of the spiral ligament and stria vascularis, the

major vascular tissue of the inner ear. Tissue (20–50 mg) was homogenized in 0.5 ml of buffer containing 10 mM Tris buffer (pH 8.0), 10 mM EDTA, 2% sodium dodecyl sulfate, and 50 mM sodium chloride. Homogenates were incubated at least 4 h with 15 μ l of proteinase K (10 mg/ml). Proteins were removed with two phenol extractions, each followed by centrifugation at $10,000 \times g$ and the drawing off of the supernatant. Residual phenol was then removed from the supernatant with an extraction with an equal volume of chloroform/isoamyl alcohol (24:1). DNA was then precipitated using 2 volumes of cold ethanol, and one tenth volume of 3 M sodium acetate (pH 5.2). This mixture was incubated at -70°C for 1 h, and then centrifuged at $10,000 \times g$ for 10 min. The pellet was dried, washed with 70% ethanol, dried again, and then redissolved in 10 mM Tris buffer (pH 8.0), 1 mM EDTA at the desired concentration. Concentrations of the DNA were determined spectrophotometrically using UV absorptions at 260 and 280 nm, and aliquots were used for PCR.

Oligonucleotide primers were designed to amplify two specific regions of the murine mtDNA genome. The cytochrome *b* gene was amplified to verify the presence of mtDNA, and the 4236 base pair deletion was amplified for quantification. The PCR reaction contains 100–150 ng of test DNA, 200 micromoles of each dNTP, 50 mM potassium chloride, 10 mM Tris-hydrochloride, 1.5 mM magnesium chloride, 0.01% (w:v) gelatin, 1 mmole of each primer, and 5 U of Taq polymerase in a final volume of 100 μ l. The thermal cycling parameters were denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 1 min, and extension at 72°C for 1 min. Amplified PCR products were separated by electrophoresis on 1% agarose gels at 96 V for 60 min, stained with ethidium bromide and visualized under ultraviolet light. After an initial amplification to look for the qualitative presence of the aging deletion, the original samples were amplified again with a [γ - ^{32}P] ATP labeled primer, and again separated by electrophoresis, this time on a 10% polyacrylamide gel. A Phosphorimager (Molecular Dynamics, Sunnyvale, CA, USA) was used to quantify the amount of radioactivity in the bands, and the total percent deletion was calculated using the amount of amplified cytochrome *b* as the total amount of mitochondrial DNA. Thresholds for each ear of each animal in both groups were analyzed with the *t*-test.

RESULTS

Enzymatic activity of cochlear SOD1

To confirm that SOD1 activity is indeed elevated in the cochlea of C57BL/6-TgN<SOD1>3Cje mice, co-

chlear proteins were separated by nondenaturing gel electrophoresis. Detection of enzyme activity by SOD1's ability to arrest free radical-mediated oxidation and precipitation of nitroblue tetrazolium [21]. Both endogenous and transgenes were expressed as active enzymes in brain, inner ear, and blood. Transgenic mice expressed a homodimer of the human transgene, a human-mouse heterodimer, and a mouse homodimer (Fig. 1, lanes 1, 3, and 5). Nontransgenic littermates expressed only the murine gene product (Fig. 1, lanes 2, 4, and 6). Note that in the native gel system used, the SOD1 bands are dimers. In transgenic tissues there is diminution of the mouse-mouse (M-M) homodimer because part of the endogenous SOD1 interacts with the transgene product to form mouse-human (M-H) heterodimers while part remains as mouse-mouse (M-M) homodimers.

Of the three tissues tested, the level of expression (activity per mg protein) was greatest for blood and lowest for cochlea. For all tissues, including cochlea, the total SOD activity was greater for transgenic mice than for nontransgenic littermates. For cochlea, the mean increase was 2.6-fold for four transgenic and four nontransgenic mice, 30 months old.

Noise exposure

Exposure of eight transgenic and eight nontransgenic littermate control mice, at 8 weeks of age, to 110 dB broadband noise for 1 h resulted in temporary threshold shifts of 32 ± 12 and 27 ± 9 dB, respectively, at 24 h postexposure. Permanent threshold shifts measured at 30 d postexposure were 13 ± 11 and 8 ± 9 dB relative to pre-exposure thresholds. At 30 d postexposure, thresholds of transgenic and nontransgenic littermates were significantly elevated (11 and 9 dB) compared to age matched mice that were not exposed to noise ($p < .001$). Hearing recovery proceeded with a similar time course for both transgenics and wild-type mice after noise exposure (Fig. 2). At no time point was there a significant difference in mean threshold due to expression of the SOD1 transgene.

Aging

To determine whether SOD may offer some protection against age-related hearing loss in C57BL/6 mice, we tested hearing at several ages between 5 and 30 weeks using click stimuli (Fig. 3). At 5 weeks of age, hearing thresholds were 20 ± 7 dB peak SPL in nontransgenic littermate control mice and 18 ± 5 dB in transgenic mice (mean \pm standard deviation, $n = 6$ and 5, respectively). For both genotypes, presbycusis developed with a logarithmic time course. By 19 weeks of age, thresholds had elevated to 38 ± 3 and 46 ± 10 dB, respectively. At no time point from 5–19 weeks of age, was there a statisti-

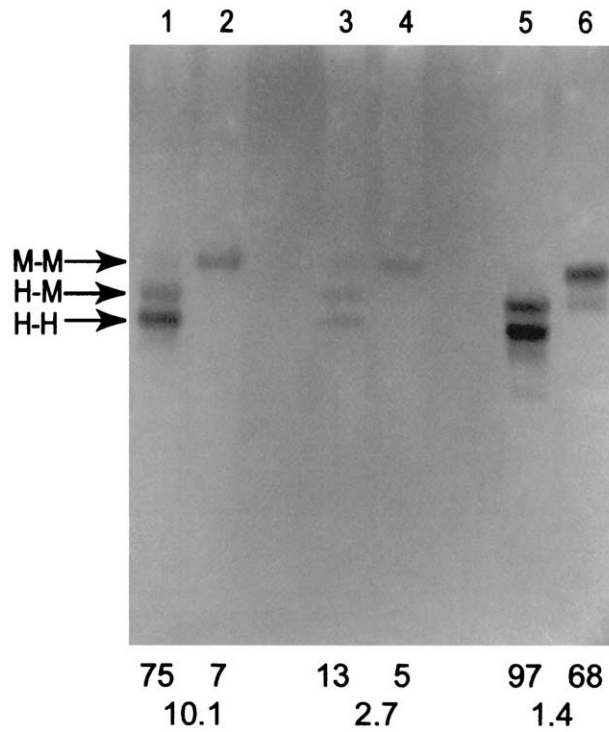


Fig. 1. Human SOD1 was expressed and active in the inner ear of transgenic mice. Forty micrograms protein from brain (lanes 1 and 2), cochlea (lanes 3 and 4), and blood (lanes 5 and 6) were separated by nondenaturing electrophoresis. The entire gel stains blue in the presence of nitroblue tetrazolium, N, N, N', N'-tetramethylethylenediamine (TEMED) and riboflavin except in gel bands containing superoxide dismutase (SOD) activity 21. The inverse image is presented to aid visualization. Transgenic mice (lanes 1, 3, and 5) express the endogenous murine homodimer of SOD1 running as a single band closest to the cathode (M-M, top), the human transgene homodimer running as a single band closest to the anode (H-H) and heterodimers of human and mouse enzyme migrating with an intermediate mobility (H-M). Littermate control mice (lanes 2, 4 and 6) express only the murine homodimer (M-M). In this gel system, hemoglobin comigrates with the H-M heterodimer (lane 6). Numbers at the bottom indicate relative total SOD1 activity (line 1) and fold increase (line 2).

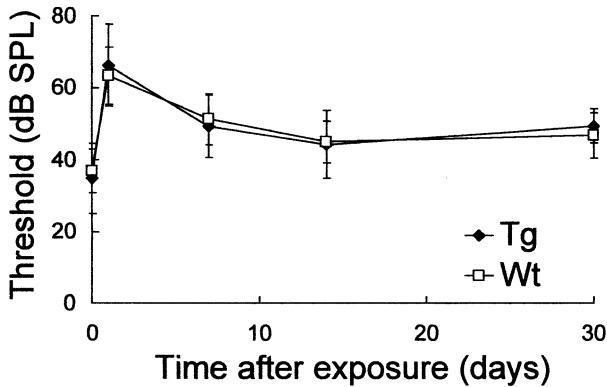


Fig. 2. Overexpression of SOD1 afforded no protection from noise-induced hearing loss. Auditory brain response click thresholds were recorded from eight transgenic mice (◆) and eight nontransgenic littermates (□) at 8 weeks of age. Following exposure to 110 dB SPL, 4–45 kHz noise for 1 h, animals were tested for hearing at 7, 14, and 30 d.

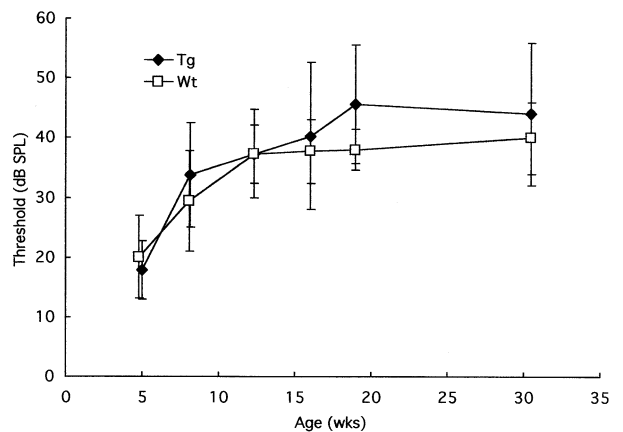


Fig. 3. SOD1 overexpression did not protect mice from age-related hearing loss. Auditory brain response thresholds were recorded from transgenic mice overexpressing SOD1 (◆) and from nontransgenic littermate controls (□) at 5, 8, 12, 16, 19, and 30 weeks of age. Mean threshold for $n = 5, 13, 6, 6, 3,$ and 2 transgenics and $n = 6, 15, 11, 7, 4,$ and 2 nontransgenic littermates \pm standard deviation are plotted as a function of age.

Table 1. Relative Level of Mitochondrial DNA Deletion

	Liver	Brain	Auditory nerve	Lateral wall
Control	0.004 ± 0.004	0.003 ± 0.001	0.005 ± 0.004	0.07 ± 0.05
Transgenic	0.003 ± 0.004	0.003 ± 0.002	0.04 ± 0.04	0.09 ± 0.09

The ratio of ^{32}P in the 4977 base pair deletion relative to that of cytochrome b DNA after polymerase chain reaction amplification of DNA from 10 wild type and 10 transgenic mice, each more than 1 year old.

cally significant difference in hearing thresholds. At age 30 weeks, thresholds were still at 40 and 44 dB SPL ($n = 2$ for both nontransgenic and transgenic mice).

Mitochondrial DNA from humans [23,24] and mice [25] is susceptible to a 4977 base pair age-related deletion. There is a high correlation of this deletion from cochlear tissue from humans with presbycusis [26] and from rats with age-related hearing loss [27]. To determine whether overexpression of SOD1 affords protection from the common 4977 base pair DNA deletion, quantitative PCR analysis was performed on tissue from liver, brain, auditory nerve and stria vascularis. The data (Table 1) show no protective effect of overexpression of SOD1. On the contrary, the percentage of deleted mitochondrial DNA in acoustic nerve increased in the transgenic mice ($p < .05$).

DISCUSSION

In the present study, cochlear SOD1 activity was found to be increased 2.6-fold over nontransgenic littermates. Despite this, and evidence for the involvement of ROS in noise-induced hearing loss (NIHL), we found no evidence for resistance of NIHL in the C57BL/6-TgN<SOD1>3Cje mouse nor for a resistance to the onset of presbycusis during the first 7 months of age.

NIHL

ROS have been implicated in cochlear damage from noise [13,15–17,28,29], ischemia-reperfusion injury [9,10,30], aging [5,26,27,31], and aminoglycoside ototoxicity [31–33]. Several approaches have been used in these studies including monitoring of the appearance of ROS in cochlear tissue and the perturbation of the antioxidant defense system. More recently, genetic tools have become available to address the role of specific gene products. Disruption of the SOD1 gene has been linked to increased susceptibility to NIHL in young mice [34], but the effect is not observed in middle-aged mice [35]. The differences in resulting permanent threshold shifts between young knockouts and wild type controls were not large, 6–14 dB. The effect was, however, significant at 5 and 40 kHz, though not at mid frequencies [34]. The click stimulus of the present study has

acoustic energy distributed over the range from below 100 to 40 kHz. However, it is possible that subtle, but important, frequency dependent differences in hearing between SOD1 overexpressing mice and nontransgenic controls may have been missed by assessment of hearing thresholds with broadband click stimuli rather than pure tones.

Sha et al. demonstrated that the transgenic mouse used in this study, C57BL/6-TgN<SOD1>3Cje [21], overexpressed SOD1 in nearly all cochlear cell types and has a resistance to the ototoxic effects of the aminoglycoside kanamycin [36]. The present study confirms an increase SOD1 activity using biochemical assays. Despite this, and evidence for the involvement of ROS in NIHL, we found no evidence for resistance of NIHL in the C57BL/6-TgN<SOD1>3Cje mouse. A rational hypothesis must account for these discrepancies.

The apparent discrepancies in the transgenic data from the literature and the present study suggest the necessity of considering a more complex involvement of antioxidant enzymes in cochlear homeostasis than previously hypothesized. Although the natures of aminoglycoside ototoxicity and NIHL are similar, the primary site of the generation of free radicals may be quite different. The generation of free radicals in aminoglycoside ototoxicity may occur in the same metabolic compartment where SOD1 resides, namely, the cytoplasm. Alternatively, free radical generation may occur in a leaky detoxifying vesicular compartment. In either case, superoxide dismutation would occur primarily in the cytoplasmic compartment and be catalyzed by SOD1. It is reasonable, however, for the primary site of superoxide anion generation in the metabolically stressed cochlea to occur within the mitochondria during and after noise exposure. This is consistent with the report of reductions of succinic dehydrogenase activity in inner and outer hair cells after noise exposure [37]. Thus, mitochondrial SOD2 may be the primary dismutase for NIHL while SOD1 serves only to remove superoxide anions that leak from mitochondria or that escape from ruptured mitochondria. In fact, SOD1 likely has such a role for leaky mitochondria because it has been localized to the annulus between inner and outer mitochondrial membranes [38]. Given this scenario, hearing loss from moderately dam-

aging noise exposures such as that used in that the study of Ohlemiller *et al.* [34] would be expectedly higher in mice with a targeted disruption of the SOD1 gene.

A variety of factors may account for why elevated expression of SOD1 in the present study was ineffective in providing further protection, beyond that of endogenous activity. First, normal levels of SOD1 may be sufficient to scavenge all of the cytoplasmic superoxide anion generated in the moderately damaging noise exposure use here and in the Ohlemiller study [34]. In this case, hearing loss that occurred in control mice may have been largely the result of the generation of damaging metabolites upstream of SOD1. A likely candidate is peroxynitrite that is rapidly formed by the reaction of the superoxide anion and nitric oxide [19]. Although peroxynitrite has not been measured directly in cochlear tissues, nitric oxide production is known to be elevated as a result of noise exposure [39]. Peroxynitrite or other metabolites upstream from SOD1 may form at a rate faster than can be compensated for with increased expression of SOD1 in C57BL/6-TgN<SOD1>3Cje mice. Alternatively, it is possible that metabolites downstream of SOD1 counteract the benefits of increased rate of removal of the superoxide anion. In this scenario, when mice overexpressing SOD1 are exposed to noise, they produce hydrogen peroxide at a rate faster than it can be removed by catalase and peroxidases. In this case, the damage from subsequent metabolism to hydroxyl radicals may be as harmful as damage from the initial generation of superoxide anions.

Exogenous application of SOD-polyethylene glycol was found to attenuate noise-induced acoustic trauma in rats [9]. The discrepancy between this finding and the lack of protection by overexpression in the present study may be related to the antioxidant system's response to the severity of the noise exposure. In the latter study, rats were exposed to 60 h of continuous broad band noise at 90 dB SPL. This severe an exposure has not been explored with the C57BL/6-TgN<SOD1>3Cje model. Alternatively, the efficacy of application of exogenous SOD may also be related to cellular compartmentation. Application of exogenous SOD, expected to act in extracellular compartments, may mimic the actions of SOD3. SOD3 is the most recently characterized member of the SOD family. Like SOD1, it is also a copper-zinc containing enzyme, but contains a signal peptide that targets it for extracellular localization [40].

Aging

Though no general conclusions about SOD1's role in presbycusis can be drawn from the present study, the results clearly show that overexpression did not provide a resistance to the onset of presbycusis in the 2–7 month

old C57BL/6. Deficiency of SOD1 in knockout mice has been shown to potentiate age-related hearing and cochlear hair cell loss [3,4]. SOD1 knockout mice, with a genetic background of a mixture of CD-1 and 129 strains, exhibited clear differences in ABR responses from 4–32 kHz at 13 months of age. In the present study, no difference in ABR response was detected between C57BL/6-TgN<SOD1>3Cje mice overexpressing SOD1 and nontransgenic littermates aged to 7 months. It is possible that overexpression of SOD1 resulted in changes in the levels of other antioxidant enzymes, such as catalase and glutathione peroxidase, that counteract the protective effects of elevated SOD1 in metabolizing free radicals. In fact, negative consequences of SOD1 overexpression have been reported. In muscle, overexpression of SOD1 was accompanied by elevated formation of OH^{\bullet} [41]. An overexpression of SOD1 and a reduced capacity to remove hydrogen peroxide may account for the significant increase in mtDNA deletions in the acoustic nerve of C57BL/6-TgN<SOD1>3Cje mice, as determined in the present study. In light of these findings, the present results warrant further investigation of the relative roles of other antioxidant enzymes in the auditory system of SOD1 overexpressing transgenic mice.

Enhanced expression of SOD1 has been shown to have protective effects against many types of tissue injury, such as ischemic and reperfusion injuries, hypoxic lung injury, brain trauma, and chemicals and drugs [42–45]. Much of our current understanding of the physiological role of reactive oxygen pathways has come about through the use of transgenic mouse models. The findings of this study were unexpected due to the apparent absence of protective effects against age- or noise-related hearing loss. The results suggest that SOD1 is not a predominant component in protection against NIHL. Further, they suggest that SOD1 may not play a major role in the onset of age-related hearing loss in this mouse model. Future studies examining overexpression and targeted deletions of antioxidant enzymes are needed to more fully elucidate the mechanisms of oxidative stress in the inner ear.

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ABBREVIATIONS

ABR—auditory brainstem response

dNTPs—deoxyribonucleoside triphosphates
EDTA—ethylenediaminetetraacetic acid
GSH—glutathione
mtDNA—mitochondrial deoxyribonucleic acid
PCR—polymerase chain reaction
ROS—reactive oxygen species
SOD—superoxide dismutase
SOD1—copper/zinc superoxide dismutase
SPL—sound pressure level