

Targeted Mutation of the Gene for Cellular Glutathione Peroxidase (*Gpx1*) Increases Noise-Induced Hearing Loss in Mice

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Received: 17 April 2000; Accepted: 16 August 2000; Online publication: 17 October 2000

ABSTRACT

Reactive oxygen species (ROS) and oxidative stress have been implicated in cochlear injury following loud noise and ototoxins. Genetic mutations that impair antioxidant defenses would be expected to increase cochlear injury following acute insults and to contribute to cumulative injury that presents as age-related hearing loss. We examined whether genetically based deficiency of cellular glutathione peroxidase, a major antioxidant enzyme, increases noise-induced hearing loss in mice. Two-month-old “knockout” mice with a targeted inactivating mutation of the gene coding for glutathione peroxidase (*Gpx1*) and wild type controls were exposed to broadband noise for one hour at 110 dB SPL. Auditory brainstem response (ABR) thresholds at test frequencies ranging from 5 to 40 kHz were obtained two and four weeks after exposure to determine the stable permanent component of the hearing loss. Depending on test frequency, (compared with controls) *Gpx1* knockout mice showed up to 16 dB higher ABR thresholds prior to noise exposure, and up to 15 dB greater noise-induced hearing loss, compared with normal control. Within the cochlear base, there was also a significant contribution of the knockout to inner and outer hair cell loss, as well as nerve fiber loss. Our results support a link between genetic impairment of antioxidant defenses, vulnerability of the

cochlea injury, and cochlear degeneration. Such impairment produces characteristics expected of some mutations associated with age-related hearing loss and offers one possible mechanism for their action.

Keywords: cochlea, hair cells, reactive oxygen species, free radical, knockout, antioxidant

INTRODUCTION

Mounting evidence implicates reactive oxygen species (ROS) in cochlear injury. Increased production of ROS has been demonstrated following cochlear ischemia (Ohlemiller and Dugan 1999), application of ototoxic compounds (Clerici et al. 1996; Hirose et al. 1997; Kopke et al. 1997), and exposure to loud noise (Yamane et al. 1995; Ohlemiller et al. 1999b). Acute impairment of antioxidant defenses promotes cochlear injury caused by these agents (Hoffman et al. 1988; Yamasoba et al. 1998; Hu et al. 1999) and, conversely, temporarily bolstering these defenses provides partial protection (Seidman and Quirk 1991; Seidman et al. 1991; Seidman and Shivapuja 1993; Garetz et al. 1994a; Garetz et al. 1994b; Hu et al. 1997). Impairment of antioxidant defenses presumably leads to increases in stress-related levels of peroxides, as well as superoxide and hydroxyl radicals, with resulting amplification of injury to lipids, proteins, and DNA of sensory cells (Halliwell 1992; Gotz et al. 1994; Evans and Halliwell 1999). Insights into the role of oxidative stress in the cochlea led to the first testable models of “metabolic” cochlear injury and have provided some of the first therapeutic approaches to preventing it.

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Chronic oxidative stress may underlie some sensorineural age-related hearing loss (ARHL) by simple application of the free radical theory of aging (Harman and Piette 1960) to the cochlea. Since oxidative injury is (or should be) remediable, and since ARHL is by far the most common form of hearing loss, identifying mechanisms that may link cumulative oxidative injury and ARHL promises significant benefits. Testing this model solely by experimentally inducing a known and realistic amount of oxidative stress for an adequate period is impractical. Gene "knockout" techniques, however, have made it possible to create a state of chronic stress by impairing the function of genes that impact cellular antioxidant defenses. Studies employing this strategy with enzymes superoxide dismutase (SOD) and glutathione peroxidase (GPx) have provided strong support for a relationship between antioxidant status and susceptibility to tissue injury (Reaume et al. 1996; Kondo et al. 1997; Yoshida et al. 1997; Schwartz et al. 1998; Spector et al. 1998; Fu et al. 1999; Jaeschke et al. 1999; Klivenyi et al. 2000). Although most of the studies performed to date do not explicitly address cumulative injury during aging, they implicitly address the genetic principles underlying why some individuals show more severe effects of aging and why some are more prone to neurodegenerative disease (Halliwell 1992; Gotz et al. 1994). Antioxidant knockout models, therefore, are useful for testing contributing factors to ARHL, especially given that a probably substantial proportion of ARHL is genetically related (Willott 1991; Schuknecht 1993; Gates et al. 1999).

There exists an attractive fit between one predicted property of at least some genetically related ARHL and genetic impairment of ROS protections: Both predict a general state of vulnerability to insults. In keeping with this prediction, experiments in mice link both ARHL-related mutations and genetic impairment of ROS defenses to increased cochlear vulnerability. The murine *Ahl* gene (Li 1992a; Erway et al. 1996; Erway and Willott 1996; Johnson et al. 1997) and probably others (Henry 1982; Ohlemiller et al. 2000b) render the cochlea especially susceptible to noise. Knockout mice for *Sod1*, the gene coding for Cu/Zn-SOD, show increased vulnerability to noise relative to wild types (Ohlemiller et al. 1999a; McFadden et al. 2000) and increased cochlear degeneration with age (McFadden et al. 1999a; McFadden et al. 1999b; McFadden et al. 2000). The impact of SOD deficiency in young adult mice (1–3 months old) was modest, yet an early and profound effect of such a mutation on hearing or cochlear vulnerability would not model the characteristics of ARHL very well.

The present study addresses the generality of the prediction that genetic impairment of ROS protections will lead to vulnerability of the cochlea, increased

cumulative cochlear injury, and ARHL. Not all genetically based ARHL is likely to act through a "vulnerability" mechanism, and counterexamples already have been reported (Yoshida et al. 2000). This study examines the effect of inactivating the gene coding for cellular GPx, which is the predominant form of glutathione peroxidase and the isoform believed to participate most widely in protecting cells from oxidative stress (Ho et al. 1997; Brigelius-Flohe 1999). GPx utilizes glutathione (GSH) to reduce reactive peroxides and thus limit lipid peroxidation and minimize the production of highly toxic hydroxyl radical via Fenton and Haber-Weiss mechanisms (Halliwell 1992; Gotz et al. 1994; Evans and Halliwell 1999). A brief account of this work has appeared previously (Ohlemiller et al. 2000a).

MATERIALS AND METHODS

Animals

All procedures were approved by the CID Animal Care and Use Committee. The study included 33 mice of both sexes, eight weeks of age (± 4 d) at the time of noise exposure. Knockout (KO, $n = 19$) and wild type mice (WT, $n = 15$) for the normal *Gpx1* gene were obtained directly from Dr. Ye-Shih Ho. *Gpx1* knockout mice are physically indistinguishable from wild types and have the same life expectancy. They exhibit no neurological, musculoskeletal, or reproductive anomalies. Young adult CBA/CaJ mice (1–2 mo, $n = 43$) were used for comparison of pre-exposure initial ABR thresholds.

To establish the *Gpx1* knockout line, 129/SVJ-derived embryonic stem cells containing a targeted mutation of *Gpx1* were transplanted to C57BL/6 donor embryos. Germline positive chimeric males were bred to C57BL/6 females, and their heterozygous offspring were crossed. Mice used in the present study were the product of homozygous matings, hence no heterozygotes or littermates of differing genotype were tested. However, the mice were offspring of four KO and two WT mating pairs formed from progeny of a single heterozygous mating pair, so that the contributions of genetic modifiers were randomized. Additional details and procedures for genotyping of parentals are given elsewhere (Ho et al. 1997).

GPx tissue typing

Livers (approximately 500 mg) were frozen in liquid nitrogen and kept at -80°C for enzyme analysis and protein concentration. They were then homogenized in 50 mM phosphate buffer (pH 7.0). GPx activity was quantified by monitoring the loss of NADPH at 340

nm in a coupled-enzyme procedure at 37°C (Prohaska 1991). Because *tert*-butyl hydroperoxide was used as the substrate, specificity of the assay for cellular GPx was not possible. However, cellular GPx is the predominant glutathione peroxidase in most tissues (Ho et al. 1997; Brigelius-Flohe 1999). One unit of GPx activity was defined as the amount of enzyme catalyzing the oxidation of 1 nmol of NADPH per minute. Specific activity for GPx is reported as units/mg protein. Protein content was determined by a modified Lowry protein assay using bovine albumin for reference (Markwell et al. 1978). Statistical tests on GPx activity were performed with SIGMASTAT (Jandel, San Rafael, CA).

Noise exposure

Noise exposures and auditory brainstem response recordings (ABRs) were performed in a foam-lined, double-walled soundproof room (Industrial Acoustics, Bronx, NY). The noise exposure apparatus consisted of a 21 × 21 × 11-cm wire cage mounted on a pedestal inserted into a B&K 3921 turntable. To ensure a uniform sound field, the cage was rotated at 1 revolution/80 s within a 42 × 42-cm metal bar frame. Motorola KSN1020A piezo ceramic speakers (four total) were attached to each side of the frame. Opposing speakers were oriented nonconcentrically, parallel to the cage, and driven by separate channels of a Crown D150A power amplifier. Noise was generated by General Radio 1310 generators and bandpassed at 4.0–45.0 kHz by Krohn-Hite 3550 filters. The overall noise level was measured at the center of the cage using a B&K 4135 0.25-in. microphone in combination with a B&K 2231 sound level meter. Noise spectrum level was determined using a Hewlett Packard 3581A wave analyzer set at 300 Hz bandwidth and is shown elsewhere (Ohlemiller et al. 1999a). Animals were exposed in pairs for 1 h at 110 dB SPL. Spot measurements indicated that levels within the cage ranged from 110 to 113 dB SPL.

ABR recordings

Auditory brainstem recordings were performed before noise exposure and 14 and 28 days after exposure. Animals were anesthetized (80 mg/kg ketamine, 15 mg/kg xylazine, IP) and positioned dorsally in a custom headholder. Core temperature was maintained at 37.5 ± 1.0°C using a thermostatically controlled heating pad in conjunction with a rectal probe (Yellow Springs Instruments Model 73A). Platinum needle electrodes (Grass, West Warwick, RI) were inserted subcutaneously just behind the right ear (active), at the vertex (reference), and in the back (ground). Electrodes were led to a Grass P15 differential amplifier

(100–10,000 Hz, X100), then to a custom amplifier providing another X1000 gain, then digitized at 30 kHz using a Cambridge Electronic Design Microl401 in conjunction with SIGNAL™ (Cambridge Electronics, Cambridge, UK) and custom signal averaging software operating on a 120-MHz Pentium PC. Sine wave stimuli generated by a Wavetek Model 148 oscillator were shaped by a custom electronic switch to 5-ms total duration, including 1 ms rise/fall times. The stimulus was amplified by a Crown D150A power amplifier and output to a KSN1020A piezo ceramic speaker located 7 cm directly lateral to the right ear, concentric with the external auditory meatus. Stimuli were presented freefield and calibrated using a B&K 4135 0.25-in. microphone placed where the external auditory meatus would normally be. Toneburst stimuli at each frequency and level were presented 1000 times at 20/s. The minimum sound pressure level required for visual detection of a response (short-latency negative wave) was determined at 5, 10, 20, 28.3, and 40 kHz using a 5-dB minimum step size. The recording noise floor was typically about 0.2 μV. Statistical tests on ABR threshold data were performed with SIGMASTAT.

Histology

Cochleas were taken for histological evaluation within one week after final ABR testing. Animals were injected with sodium pentobarbital (60 mg/kg IP) and perfused transcardially with cold 2.0% paraformaldehyde/2.0% glutaraldehyde in 0.1M phosphate buffer. Each cochlea was rapidly isolated and immersed in the same fixative, and the stapes was immediately removed. Complete infiltration of the cochlea by fixative was ensured by making a small hole at the apex of the cochlear capsule and gently circulating the fixative over the cochlea using a transfer pipette.

One cochlea from each animal was used for hair cell counts; the other was used to assess nerve fiber loss. For hair cell counts, cochleas were transferred to phosphate-buffered saline (pH 7.4), dissected into half-turn segments, and stained in Harris' hematoxylin solution (McFadden et al. 1999b). Stained segments were whole-mounted in glycerin on glass slides. Surface preparations were examined using light microscopy at 400X magnification. The numbers of inner (IHC) and outer hair cells (OHC) were counted in successive segments (0.24 mm average length) along the organ of Corti, beginning at the apex. The percent of hair cells missing was determined by comparing hair cell counts of individual mice to lab standards for normal CBA mice. For nerve fiber counts, fixed cochleas were decalcified (Decal, Baxter Scientific Products, Deerfield, IL) and embedded in Epon 812 resin. Serial sections (3 μm thick) were made parallel

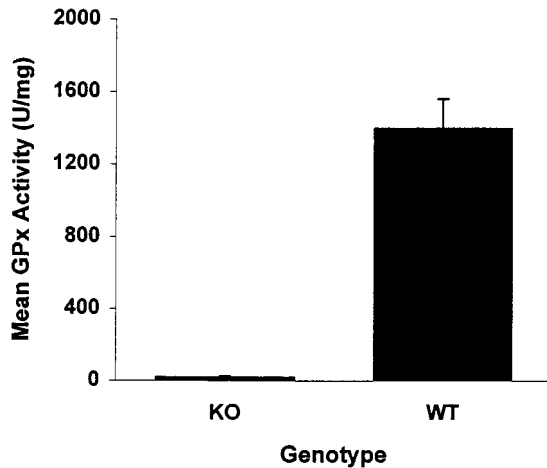


FIG. 1. Mean (+SD) GPx activity in liver of *Gpx1* wild type (WT) and knockout (KO) mice. One unit of activity corresponds to the reduction of 1 nmol NADPH/mg protein/min. Differences by genotype are significant (*t* test, $p << 0.001$).

to the modiolar axis. Sections were stained with toluidine blue and examined under the light microscope. All nerve fibers in three apical and three basal habenuae were counted and averaged for each animal. No attempt was made to distinguish among type I and type II afferent or efferent fibers. Statistical tests on hair cell and nerve fiber data were performed with SPSS.

RESULTS

Tissue typing for GPx activity

Figure 1 compares mean GPx activity in whole liver of knockout and wild type mice. Specific activity is expressed in nanomoles of NADPH reduced per minute per milligram of protein in each sample. Enzyme activity showed perfect correspondence with genotype with no overlap, and the difference in activity by genotype was highly significant (*t* test, $p << 0.001$).

Pre- and postexposure thresholds

Figure 2 shows ABR thresholds by genotype prior to noise exposure. Thresholds from similar-aged normal CBA mice have been included for comparison. Even before noise exposure, and despite the fact that the animals were only two months old, both WT and KO mice exhibited high-frequency hearing loss compared with CBAs. Thresholds in both genotypes averaged above 80 dB SPL at 40 kHz and above 55 dB SPL at 28.3 kHz, while typical thresholds in normal CBA mice at these frequencies are below 40 dB SPL. Some degree of initial hearing loss was evident at frequencies as low

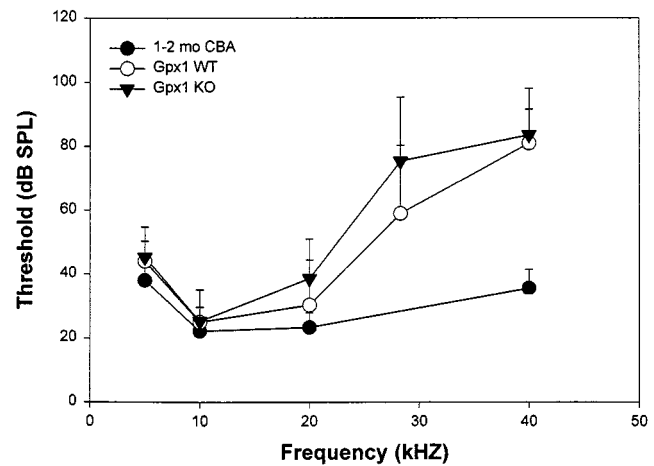


FIG. 2. Mean (+SD) hearing sensitivity in WT and KO mice determined by ABR thresholds prior to noise exposure. There are significant differences by genotype (two-way ANOVA, $p = 0.006$). Similar-aged CBAs are shown for comparison. CBAs are from another study (Ohlemiller et al. 2000b) and were not tested at 28.3 kHz.

as 20 kHz, although it appeared less than 10 dB in WT. For both genotypes, thresholds at 40 kHz were considered too elevated to be informative so that statistical tests were applied only to results obtained at 5–28.3 kHz.

GPx deficiency led to significant elevation of ABR thresholds prior to noise exposure (two-way ANOVA, $p = 0.006$), with interactions between genotype and test frequency. *Post hoc* testing (Student–Newman–Keuls (SNK), $p < 0.05$) detected a significant difference only at 28.3 kHz, where the magnitude of the effect was about 16 dB (Table 1).

Size of effect of *Gpx1* knockout Figure 3 compares ABR thresholds by genotype two and four weeks after noise exposure. Permanent noise-induced threshold shifts were stable by two weeks. Because statistical analyses gave similar results at both time points, only four-week results are described. *Gpx1* KO mice showed significantly greater threshold elevation after exposure than WTs (two-way ANOVA, $p < 0.001$). *Post hoc* tests (SNK, $p < 0.05$) revealed significant differences at 20 and 28.3 kHz, where differences by genotype ranged from 15 to 23 dB. Allowing for initial threshold differences, the contribution of GPx deficiency to noise-induced hearing loss (NIHL) ranged from ~0 to 15 dB across all test frequencies (Table 1).

Although a significant main effect of genotype was detected, examination of the distribution of ABR thresholds by test frequency (Fig. 4) suggests that analysis by *Gpx1* genotype alone may have underestimated the impact of GPx deficiency. Particularly after exposure, threshold distributions at 10, 20, and 28.3 kHz were marked by high interanimal variance and the suggestion of multiple modes, although these were not sufficiently distinct to permit further analysis. One

TABLE 1

Mean threshold (dB SPL) at	Size of effect <i>Gpx1</i> knockout						Estimated contribution of KO to NIHL (dB)
	Pre-exposure ^a			4 weeks post ^a			
	WT	KO	Difference (dB)	WT	KO	Difference (dB)	
5 kHz	44.0	45.3	1.3	51.7	51.8	0.1	-1.2
10 kHz	25.0	25.3	0.3	40.7	53.0	12.3	12.0
20 kHz	30.3	38.6	8.3	51.9	74.8	22.9	14.6
28.3 kHz	59.0	75.4	16.4	75.1	90.0	14.9	-1.5

^aKO = knockout, WT = wild type for *Gpx1*.

^bSignificant by *post hoc* comparisons.

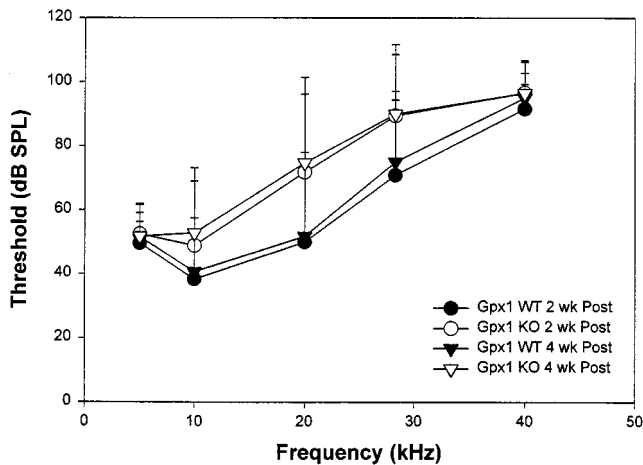


FIG. 3. Mean (+SD) hearing sensitivity in WT and KO mice compared two and four weeks post exposure. Noise-induced threshold shifts were stable. There are significant differences by genotype (two-way ANOVA, $p < 0.001$).

interpretation of this distribution is the presence of additional unlinked genes that independently modulate noise vulnerability. Alleles of these genes would vary within *Gpx1* genotype since the mice were not inbred. However, increased variance in noise-induced threshold shifts relative to pre-exposure thresholds can be observed even in inbred and F1 hybrid mice (Ohlemiller et al. 2000b; Ou et al. 2000a) and may be a result of environmental factors.

Hair cell loss

Noise-exposed *Gpx1* knockouts showed greater hair cell loss than wild type controls. A significant main effect of genotype was found for both IHCs (two-way ANOVA, $p = 0.016$) and OHCs ($p = 0.035$), with interactions between genotype and cochlear region. Both KO and WT mice had hair cell loss, essentially all of which was confined to the most basal 40% of the cochlea (Fig. 5.). This basal loss was more pronounced in GPx-deficient mice, however, for IHCs

(*post hoc* one-way ANOVA, $p = 0.013$) and OHCs ($p = 0.021$). According to a recent frequency-place map for the mouse (Ou et al. 2000b), the basal 40% of the cochlea should correspond to our three highest test frequencies (20–40 kHz), with 10 kHz represented about 47% of the distance from the apex. Figure 6 presents an example of the use of hematoxylin staining to enhance recognition of hair cells and facilitate cell counts. Surface preparations taken from the lower cochlear base of an unexposed normal mouse (Fig. 6A) are compared with that taken from a noise-exposed *Gpx1* knockout mouse (Fig. 6B). Severe hair cell loss was sometimes associated with loss of the organ of Corti in restricted regions, but this was observed in both WT and KO. Thus, hair cell loss, and not the extent of degeneration, was the primary distinguishing feature between the KO and WT organ of Corti.

Nerve fiber loss

GPx deficiency also led to increased loss of cochlear nerve fibers in the exposed mice. A significant main effect of genotype was found (two-way ANOVA, $p = 0.002$), with interactions between genotype and cochlear region. Both WT and KO mice showed significantly more nerve fibers per habenula in the apex than in the base (two-way ANOVA, $p < 0.001$) (Fig. 7). *Gpx1* KO mice showed nearly a 50% greater reduction in fibers/habenula than WT, however (*post hoc t* test, $p = 0.002$). Figure 8 illustrates this difference in representative exposed WT and KO mice as it would appear for quantitation. Evacuated spaces within the bony channels suggest nerve fiber loss in both animals, but they are much more apparent in the knockout.

DISCUSSION

Gpx1 knockout model

Even prior to noise exposure, both *Gpx1* knockouts and wild types showed hearing loss at the highest test

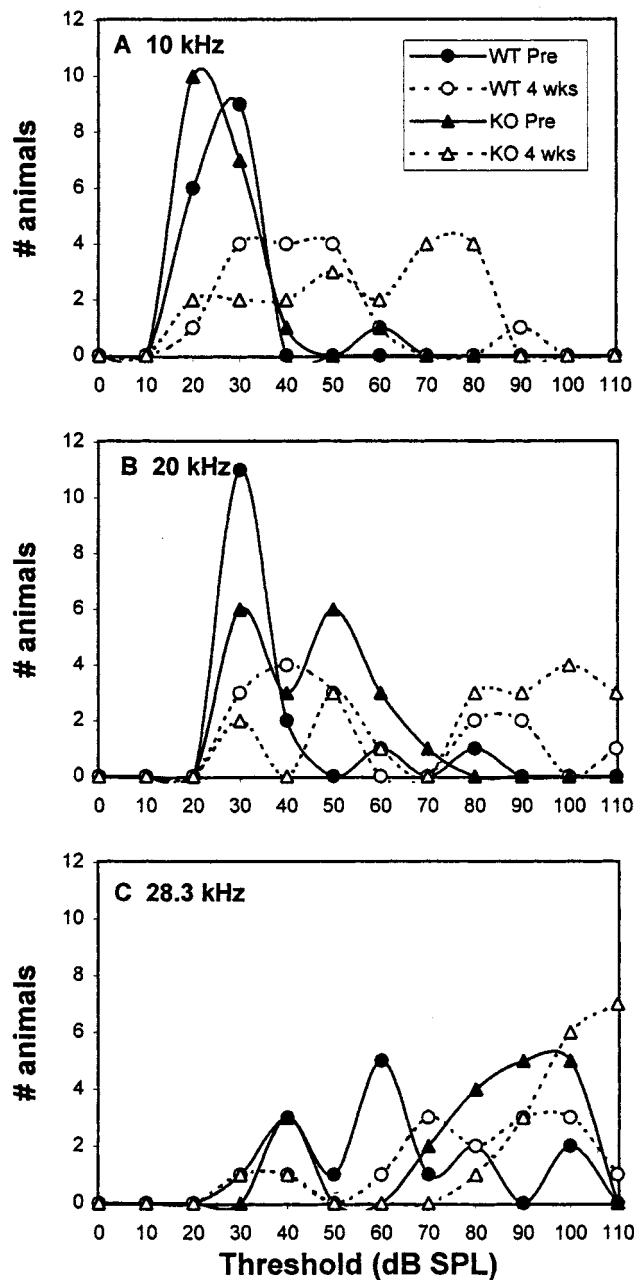


FIG. 4. Distribution of ABR thresholds by genotype before noise exposure and four weeks later at 10, 20, and 28.3 kHz. Thresholds were assigned to 10-dB bins. Lines are curvilinear fits.

frequencies. This hearing loss extended to lower frequencies in KOs, however, and was up to 16 dB greater in the KOs, depending on test frequency (Table 1). In neither KO nor WT mice was the initial hearing loss explicitly demonstrated to be age-related, yet this is a reasonable interpretation of this finding. The animals used in the study are a cross of C57 and 129 inbred strains, both of which show early and progressive cochlear degeneration that includes hair cell loss (Henry 1983; Li 1992b; Zheng et al. 1999). The absence of genetic complementation in our hybrids

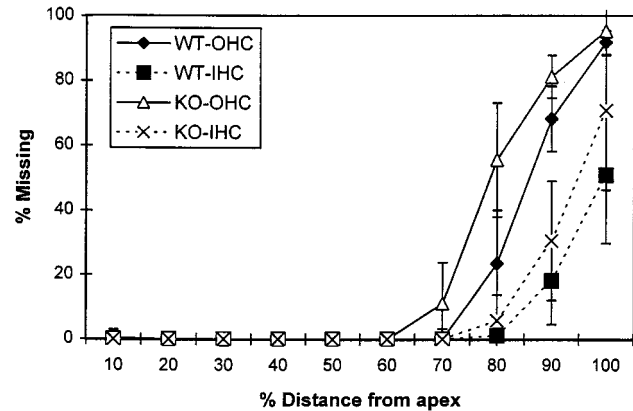


FIG. 5. Hair cell loss by genotype determined 4–5 weeks postexposure in hematoxylin-stained surface preparations. There were significant differences by genotype for both inner (two-way ANOVA, $p = 0.016$) and outer hair cells ($p = 0.035$). IHC = inner hair cell, OHC = outer hair cell.

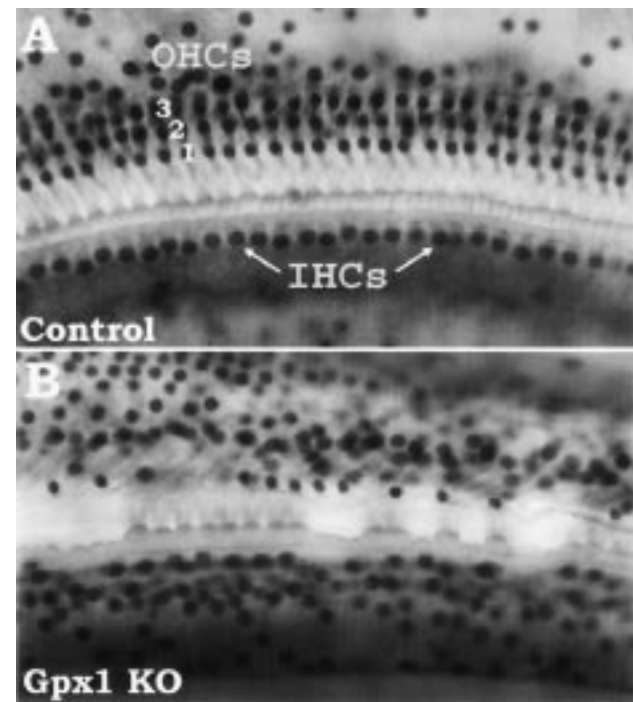


FIG. 6. Representative surface preparations from (A) a normal control mouse and (B) noise-exposed *Gpx1* KO as they are viewed for hair cell counts. IHC = inner hair cell, OHC = outer hair cell.

suggests that both may carry the *Ahl* mutation, known to be carried by C57BL/6 mice (Johnson et al. 1997). The fact that KOs and WT mice exhibited the largest initial threshold differences at the highest test frequencies suggests that GPx deficiency may magnify the influence of *Ahl* (or another similar-acting gene). Any hearing loss based on such interactions is very likely to be progressive and, thus, age-related.

GPx-deficient mice also showed increased susceptibility to NIHL. Although this effect was clear, it was

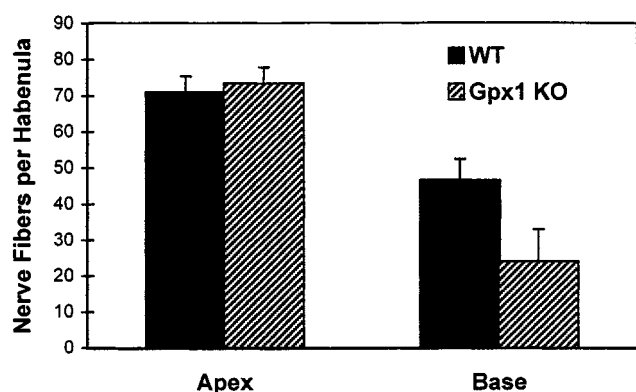


FIG. 7. Comparison of nerve fiber density in the cochlear apex and base of WT and KO mice. Density of fibers in the apex and base differed significantly (two-way ANOVA, $p < 0.001$) for both genotype. Knockout mice showed significantly fewer fibers within the base (t test, $p = 0.002$).

modest and it varied with test frequency. The contribution of the knockout to hearing loss ranged from ~0 dB at 5 kHz to an estimated 15 dB at 20 kHz (Table 1). Apparent age-related hearing loss at 28.3 kHz, also attributable to GPx deficiency, probably reduced the noise-induced component of hearing loss at this frequency. Given the increasing impact of the KO with frequency (Table 1), an effect of the KO on NIHL would likely otherwise have been found at 28.3 kHz also. Conclusions regarding the extreme cochlear base were hampered by a high-frequency hearing loss and cochlear degeneration common to both genotypes. If GPx deficiency promotes injury that may present as ARHL, then it is also true that no clear distinction between the influence of GPx deficiency on ARHL and NIHL can be assigned in our data. Initial high-frequency hearing losses could have been caused in part by inadvertent noise exposure (e.g., animal facility noise, shipping) or other influences.

GPx deficiency was also associated with greater hair cell and nerve fiber loss, at least within the cochlear base. Because cell counts were not obtained from unexposed animals, this degeneration cannot be attributed with certainty to the effects of aging versus noise. Note, however, that the spatial patterns of hair cell losses observed in both KO and WT mice are more consistent with pre-exposure hearing thresholds than with postexposure thresholds. OHC losses in KO mice extended 40% of the distance from the base, consistent with initial hearing losses at 20 kHz and above (Ou et al. 2000b). Similarly, OHC losses in WT mice extended only about 30% of the distance from the base, in keeping with their near-normal thresholds at 20 kHz. By contrast, hair cell loss did not extend far enough apically to account well for the observed NIHL in either KO or WT mice. Therefore, it seems likely that it was the pre-existing “age-related” component of hair cell

and nerve fiber loss that was increased by GPx deficiency, as reflected in our measurements. It is not surprising that hair cell loss did not correspond well with the frequency range of NIHL in our mice. Several reports have described the general inability of hair cell loss to “explain” NIHL in mice (Fowler et al. 1995; Ohlemiller et al. 1999b; Yoshida et al. 1999; Ou et al. 2000a), although there may be a better correspondence in young adult mice (Ohlemiller et al. 2000b). To account for NIHL, other nonlethal injuries to hair cells (e.g., Yoshida et al. 1999) must be invoked.

Role of genetic background

The impact of genetic background in this and similar studies merits discussion. First, it is important to distinguish between the two major types of influence that genetic background may have. Most knockout and transgenic mouse studies involve hybrid mice, reflecting the methodological convenience of inserting genes into certain strains that otherwise may be disadvantageous by other criteria (Crusio 1996; Lathe 1996; Choi 1997). Repeated sibling crosses over 10–20 generations eventually yields the genetic standardization of a new mouse substrain or strain, while backcrossing to one of the parental strains more rapidly moves the transgene to a congenic background that asymptotically approaches that parental strain (Green 1975). It is in the transition that our animals—and those used in the vast majority of similar studies—are produced and evaluated. Such animals are neither completely hybrid nor inbred. Both hybrid and inbred models have their advantages and drawbacks. However, as we shall explain, most studies are subject to some limitations of both.

Hybrids are heterozygous at many loci, approximating the situation in humans, and provide a model in which an effect of the transgene or KO should be seen if the gene of interest is truly important. Hybrid models involving outbred strains, such as CD1, can be even more demonstrative in this regard. However, studies seeking to uncover subtle effects, such as the current study, are subject to increased variance in the data obtained. This variance is caused by genetic modifiers that vary from animal to animal, independent of the gene under study. In such cases, any effects of the transgene or KO may be partially obscured and thereby underestimated. This limitation is unlikely to lead to inflated effects. However, negative results must be carefully interpreted. If fortune prevails, the distribution of the data may reveal an order, such as discernible peaks, allowing some sources of variance in the data to be factored out. In our previous study of *Sod1* KO mice (Ohlemiller et al. 1999a), this feature of the data made it possible to compare subsets of KO and WT mice so that a more accurate determination of the

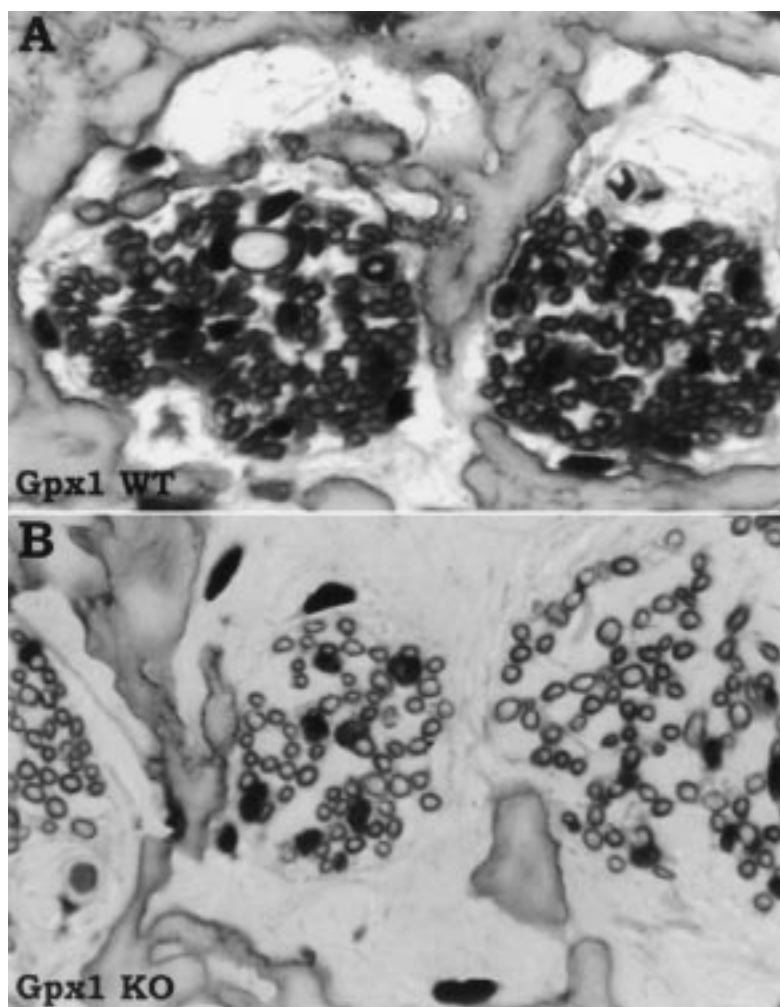


FIG. 8. Light photomicrographs of representative habenula perforata in the cochlear base of exposed (A) WT and (B) KO mice as they are viewed for nerve fiber counts. The plane of section is parallel to the modiolar axis.

effects of SOD deficiency could be obtained. In the present study, the data were similarly marked by a high degree of variance within *Gpx1* genotype, and, again, the primary contributor was probably the influence of additional genes. However, there was no clear underlying order in the threshold distribution that might have permitted the separation of animals for comparison by subgroups. The likely result of analysis by *Gpx1* genotype alone was an underestimate of the effects of the knockout. Another possible factor is the increase in variance of thresholds that is often observed following noise exposure, even in inbred and F1 hybrid mice (Ohlemiller et al. 2000b; Ou et al. 2000a). This may reflect differences in susceptibility resulting from unknown and unintended environmental conditioning or slight differences in the noise exposure itself.

Inbred models eliminate obscuring of results caused by genetic heterogeneity of the sample. However, few inbred strains will be found to be “neutral” in terms of their impact on the observed phenotype of a KO or transgenic model, and no strain can be assumed *a priori* to be superior for a given study (Crusio 1996; Lathe 1996; Choi 1997). Cases abound in the

literature of murine mutations whose associated phenotype varies, depending on the strain. For example, the *rd3* retinal degeneration mutation gives rise to widely varying phenotypes on different strains (Hecklively et al. 1993). The *tub* mutation, which arose in C57BL/6, has generated much interest for its pleiotropic effects on hearing, vision, and body weight (Ohlemiller et al. 1997). Yet, its presence might go undetected on a different inbred strain (Ikeda et al. 1999). Usually, the genetic modifiers that underlie such strain dependence are unknown. Even if genes known to be present are potential modifiers (by virtue of a known impact on the trait that is being measured), the nature of their interaction with the KO or transgene cannot be predicted. The implication of these observations is that no one model should be assumed to provide a definitive answer regarding the effect of any KO or transgene. Instead, any one model indicates one possible outcome. Within this framework, our present and previous results in *Gpx1* and *Sod1* KO mice support a principle whereby genetic impairment of antioxidant defenses appears capable of promoting cochlear injury and ARHL.

Hybrid backgrounds are not immune to issues of strain dependence. Crossing inbred strains leads to somewhat limited genetic variability since the parent strains are homozygous at essentially all loci. Because of this, some or all animals in the sample may be homozygous for mutations that could influence results. In the present study, as well as our study of *Sod1* knockout mice (Ohlemiller et al. 1999a), the combination of two strains used in generating the animals led to an initial high-frequency hearing loss. Our analysis of *Sod1* KOs indicated that mutation(s) responsible for this hearing loss acted independently of the effects of SOD deficiency. By contrast, in *Gpx1* KOs the effect of the knockout may be to amplify the effect of *Ahl* or another mutation, based on the observed spatial pattern of hearing and cell loss. Like most other investigators, we did not have the luxury of picking from many KO models but used the models that were available. These models were developed on backgrounds chosen not for their hearing ability but for their advantages in gene manipulation, as was stated. Nearly every knockout model for which basic auditory sensitivity has been adequately described involves the inbred C57 and 129 strains and the outbred CD1 strains and features age-related hearing loss (Shone et al. 1991; Zheng et al. 1999). Given a choice, we would not have picked such models for our initial studies. However, from the foregoing, we would strongly argue that these models are not inherently any less suitable than other models. Indeed, it would be desirable to examine the effect of genetic impairment of antioxidant enzymes explicitly on one or more backgrounds that include deafness genes. As it happens, that is where we began.

GPx, glutathione, and oxidative cochlear injury

Hydrogen peroxide, the primary substrate of cellular GPx, participates in both Fenton and Haber Weiss reactions, the two key reactions that lead to the formation of the highly toxic hydroxyl radical (Halliwell 1992; Gotz et al. 1994; Evans and Halliwell 1999). By its action in regulating cellular levels of peroxide, GPx plays a critical role in minimizing the production of hydroxyl radical. Although there may be species differences (Lautermann et al. 1997), GPx appears present at comparatively high levels in the cochlea (Pierson and Gray 1982). To sustain its activity, cellular GPx requires selenium and glutathione, so that deficiencies in these as well as in GPx itself might produce similar effects. One recent study involving GSH depletion prior to noise exposure, combined with the present results, permits comparison of the effects of chronic and acute impairment of GSH-dependent antioxidant defenses. That study (Yamasoba et al. 1998) involved a different noise-exposure paradigm and a different

animal model (guinea pig), yet it produced results strikingly similar to those of the present work. Among the features reproduced were the approximate magnitude of the effect in dB, an increased effect at higher frequencies, and hair cell loss. This is particularly surprising, since depletion of GSH should cripple not only GPx but also GSH transferases, which have some overlapping actions with GPx (Ketterer 1998), as well as nonenzymatic antioxidant functions of GSH itself. Other studies have also shown that GSH depletion enhances ototoxic injury, which probably also reflects impairment of each of these functions (Hoffman et al. 1988; Hu et al. 1999).

Significance of GPx versus other antioxidant enzymes

Based on models examined so far, the impact of SOD deficiency appears generally comparable to that of GPx deficiency. Both contribute modestly to cochlear susceptibility and apparent ARHL without lethality, CNS pathology, or dramatic hearing loss. It is not clear why the impact of SOD deficiency should be similar to that of GPx deficiency with respect to the magnitude of their effects on NIHL. SOD and GPx perform different functions. SOD deficiency may yield modest results because SOD removes a toxic radical (superoxide) yet produces another reactive intermediate (peroxide). Both substrate and product take part in the production of hydroxyl radical. Superoxide also contributes to the production of peroxynitrite (Halliwell 1992; Gotz et al. 1994; Evans and Halliwell 1999), whose role in noise injury is not well understood. In sum, both decreases and increases in SOD activity may be offset. The primary action of GPx—removing a toxic intermediate—would be expected to be more straightforward. In addition, GPx may remove peroxynitrite (Sies et al. 1997), potentially compounding its beneficial actions. Experiments with transgenic overexpressors of SOD and GPx, or acute addition of these enzymes, have shown a greater effect of GPx than either SOD or catalase (Weisbrot-Lefkowitz et al. 1998). Nevertheless, there may exist sufficient redundancies in antioxidant protections so that the effect of removing any one component is partly mitigated. There are multiple isoforms of both of these enzymes, although these isoforms are present in much smaller amounts and located in different compartments (Carrlson et al. 1995; Li et al. 1995; Brigelius-Flohe 1999). There is also some duplication of function by other enzymes such as GSH transferases (which detoxify some types of hydroperoxides) (Ketterer 1998) and catalase. Any compensation by other enzymes apparently does not, however, involve up-regulation of these in the absence of cellular GPx (Cheng et al. 1997; Ho et al. 1997). Finally, GSH does not require the presence of GPx to

perform a significant antioxidant role. The fact that the current GPx results are similar to those obtained in acute experiments involving impairment of GSH-dependent antioxidant systems (Yamasoba et al. 1998) raises the possibility of an upper limit to the effect of crippling any subset of antioxidant protections.

Relevance to age-related hearing loss

Genetic impairment of antioxidant protections can lead to both age-related and noise-induced hearing loss, apparently by conferring a state of vulnerability on the cochlea (McFadden et al. 1999a; Ohlemiller et al. 1999a; McFadden et al. 2000). Knockout models of this type resemble the expected interplay of genes and some human acquired hearing loss, whereby genetically susceptible individuals sustain cumulative cochlear damage that presents as ARHL. That ARHL-related genes exist, and that some of these may work in this manner, has been speculated (Henry 1982; Willott 1991; Schuknecht 1993). The murine *Ahl* mutation provides a specific example (Li 1992a; Erway et al. 1996; Erway and Willott 1996; Johnson et al. 1997), although there is no evidence that it directly affects antioxidant defenses. Thus, it is possible that genetic impairment of antioxidant defenses underlies some ARHL in humans. Most of the studies dealing with oxidative injury to the cochlea have focused on a handful of enzymes, such as GPx, SOD, and GSH transferases. Certainly, mutations that impair specific isoforms of these may be involved. Just as likely, however, are mutations of an unknown number of genes that impact cellular redox status or coordinately regulate antioxidant defenses.

ACKNOWLEDGMENTS

We thank Jaci Lett for assistance with figure preparation and Dr. Barbara Bohne for comments on the manuscript. Supported by the Edward Mallinckrodt, Jr. Foundation (KKO), NIH R01 DC03454 (KKO), and NIH R01 HL56421 (YSH).

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