Paraoxonase and Superoxide Dismutase Gene Polymorphisms and Noise-Induced Hearing Loss

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Background: Noise-induced cochlear epithelium damage can cause hearing loss in industrial workers. In experimental systems, noise induces the release of free radicals and may damage the cochlear sensorial epithelium. Therefore, genes involved in regulating the reactive oxygen species manganese-superoxide dismutase (SOD2) and the antioxidant paraoxonase (PON) could influence cochlea vulnerability to noise. We evaluated whether susceptibility to noise-induced hearing loss (NIHL) is associated with SOD2, PON1, and PON2 polymorphisms in workers exposed to prolonged loud noise.

Methods: We enrolled 94 male workers from an aircraft factory in the study. The SOD2 gene was screened by denaturing reversed-phase HPLC, and the PON1 (Q192R and M55L) and PON2 (S311C) polymorphisms were analyzed by PCR amplification followed by digestion with restriction endonucleases.

Results: Three known (A16V, IVS3-23T/G, and IVS3-60T/G) and two new SOD2 polymorphisms (IVS1/H11545-8A/G and IVS3/H11545-107T/A) were identified. Regression analysis showed that PON2 (SC/CC) [odds ratio (OR) = 5.01; 95% confidence interval (CI), 1.11–22.54], SOD2 IVS3-23T/G and IVS3-60T/G (OR = 5.09; 95% CI, 1.27–20.47), age (OR = 1.22; 95% CI, 1.09–1.36), and smoking (OR = 49.49; 95% CI, 5.09–480.66) were associated with NIHL. No association was detected for PON1 (QQ+RR) and PON1 (LL) genotypes.

Conclusions: Our data suggest that SOD2 and PON2 polymorphisms, by exerting variable local tissue antioxidant roles, could predispose to NIHL. However, caution should be exercised in interpreting these data given the small sample size and the difficulty in matching cases to controls regarding the overwhelming risk factor, i.e., smoking at least 10 cigarettes/day.

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Cochlear epithelium damage attributable to noise is a major cause of permanent hearing loss in industrial workers. It has been demonstrated in experimental systems that noise, by inducing the local release of free radicals, may damage the cochlear sensorial epithelium (1). Consequently, genes involved in the regulation of reactive oxygen species, such as superoxide dismutase (SOD)6 genes, may affect the vulnerability of the cochlea to noise-induced hearing loss (NIHL) (2).

SOD enzymes catalyze the conversion of superoxide radicals to hydrogen peroxide: manganese SOD within the mitochondrion, copper-zinc SOD in the cytosol, and extracellular SOD in the extracellular compartment (3). Manganese SOD is a homotetramer, and each of its subunits is encoded by the SOD2 gene on chromosome 6q25. The gene spans five exons and produces a 222-amino acid protein whose first 24 amino acids represent the mitochondrial targeting sequence (4, 5). A limited number of polymorphisms have been identified in the SOD2 gene, C47T being the most widely studied. C47T is located at position 16 in the mitochondrial targeting sequence.
sequence and causes the replacement of an alanine with a valine (A16V) (3). It has been studied in association with various diseases, with discordant results (6–9). To date, three intronic, non-disease-related SOD2 polymorphisms have been detected (10).

To screen the PCR products of the SOD2 gene, we developed a denaturing reversed-phase HPLC (DHPLC) procedure that is highly sensitive (96%) and less expensive than the reference method of direct sequencing (11). An additional advantage of the DHPLC procedure is that the post-PCR analysis can be automated, thereby saving time.

Paraoxonases (PONs) exert antioxidant activity and may protect against diseases such as atherosclerosis, diabetes, Alzheimer dementia, and Parkinson disease (12–16). The PON gene family consists of PON1, PON2, and PON3 on chromosome 7q21-q22 (17). PON1 and PON3 are closely associated with apolipoprotein A-1 in HDL and may enhance its antiatherosclerotic properties (18). PON2 is ubiquitously expressed in tissues throughout the body (19) and may exert its antioxidant effect at a cellular level. Polymorphisms have been detected at codons Q192R and M55L in the PON1 gene and at codon S311C in the PON2 gene (20, 21). Rare PON3 point mutations (<1%) have been detected in apparently healthy heterozygotes (18). PON1 (55) L, PON1 (192) R, and more recently PON2 (311) C variants have been implicated in the oxidative damage associated with the pathogenesis of neurodegenerative diseases such as Alzheimer disease and Parkinson disease (22, 23).

The aim of this study was to determine whether susceptibility to NIHL is related to SOD2, PON1, and PON2 polymorphisms in workers employed at the Alenia Aeronautica aircraft factory. We also investigated routine biochemical indices and total radical-trapping antioxidant plasma (TRAP) activity to test their association with hearing loss evaluated through audiometric tests.

Materials and Methods

PARTICIPANTS

The overall cohort consisted of 252 men from the Campa-

nia Region working at Alenia Aeronautica (Pomigliano D’Arco, Naples, Italy); the age range was 29–58 years. All men were exposed to sound pollution ranging from 61.2 decibels [dB (A)] to 98.0 dB (A). The following information was collected for all participants: medical histories, presence of metabolic diseases, and lifestyle and smoking habits [smokers (>10 cigarettes/day) and combined non-smokers/light smokers (<10 cigarettes/day) because the medical records did not distinguish light smokers from nonsmokers]. All participants underwent an audiometric examination. Exclusion criteria were presence of cardio-

vascular events, diabetes, hyperlipidemia, and unmeasurable audiometric data because of poor collaboration by the participant. The inclusion criterion was exposure to a mean (SD) noise level equivalent to 92.4 (4.1) dB (A) for 20 years and use of the same noise-protection equipment.

Ninety-four workers of 252, selected on the basis of the above stringent criteria, were enrolled in the study and underwent the genetic, biochemical, and audiometric analysis. The study was approved by the Ethics Committee of our Medical School, and informed consent was obtained from each individual.

AUDIOMETRIC EXAMINATION

Participants underwent otoscopy and tonal audiometric examination in a sound isolation cabinet. They were exposed to pure tones at 125, 250, 500, 1000, 2000, 3000, 4000, 6000, and 8000 Hz via earphones (air conduction) and pure tones at 250, 500, 1000, 2000, 3000, and 4000 Hz via a vibrator pressed against the mastoid portion of the temporal bone (bone conduction). The faintest pure tone that an individual could hear at each frequency was plotted on a graph (audiogram), and the hearing level was established. Normal hearing was diagnosed as follows: hearing any tone ≥25 dB, according to the Occupational Safety and Health Administration [46 FR 4078 (1981a) and January 1, 2003 (66 FR 52031–52034)]. According to audiometric results, 63 individuals had NIHL and 31 had normal hearing. Fig. 1 shows the mean audiograms of both groups.

DNA ANALYSIS

Genomic DNA was extracted from peripheral blood samples by standard procedures (24). The SOD2 gene exons, including intron/exon junctions, of each DNA sample were amplified with PCR using five primer pairs designed based on the human SOD2 sequence (EMBL accession no. S77127). The PCR products were screened with a DHPLC procedure (Wave System 3500; Transgenomic) devised in our laboratory. The primer sequences, PCR product sizes, PCR annealing temperatures, and DHPLC conditions are listed in Table 1. The primers for exon 2 are described elsewhere (8). In each run, six control samples (one wild-type and five bearing polymorphisms) were tested together with the DNA of the study participants. The control DNAs had been typed previously by sequence analysis with fluorescent dye-terminator cycle sequencing on an automated sequencer (ABI 373A; Applied Division, Perkin-Elmer).

Here we use the den Dunnen and Antonarakis nomenclature (25) for SOD polymorphisms. The PON1 polymorphisms Q192R and M55L and the PON2 polymorphism S311C were determined by PCR amplification followed by digestion with restriction enzymes Alul, NlaIII, and DdeI, respectively (20, 21). The PCR products were resolved on a 4% metaphor gel and visualized by staining with ethidium bromide. PON genotypes were assessed independently by two observers. In each PON polymorphism, three control DNA samples (preliminarily verified by sequence analysis) were tested at the same time as the DNA samples from study participants.
SAMPLES AND BIOCHEMICAL MEASUREMENTS

Venous blood was sampled from all participants after an overnight fast. Serum total cholesterol, triglycerides, and glucose were measured enzymatically with a standard technique on an automated analyzer (Hitachi 747; Boehringer Mannheim). The HDL-cholesterol concentration was determined enzymatically by measuring cholesterol in the supernatant after precipitation with phosphotungstate. LDL-cholesterol was calculated according to the Friedewald formula.

TRAP activity was measured with a spectrophotometric end-point method on a Cobas centrifugal analyzer (Hoffmann-La Roche) (27). The synthetic water-soluble tocopherol analog Trolox™ (Hoffmann-La Roche) was used for calibration. The intra- and interassay imprecision coefficients (CVs), evaluated on a plasma pool, were 1.7% and 3.2%, respectively. TRAP activity is indicative of the antioxidant defense of plasma against free radicals and is based particularly on albumin, urate, ascorbate, bilirubin, α-tocopherol, and β-carotene.

STATISTICAL ANALYSIS

Allele frequencies were calculated by allele counting, and the departure from Hardy–Weinberg equilibrium was evaluated by the χ² analysis. Linkage disequilibrium between the different polymorphisms in each PON1 and SOD2 gene was evaluated, and its significance was tested by the Fisher test. Associations of the PON1, PON2, and SOD2 gene polymorphisms with continuous variables were tested by one-way ANOVA, and with categorical variables by the χ² test. Bonferroni correction for multiple comparisons was performed when required (28).

To check for risk genotypes, we combined PON1 (55) (M/M vs L/L), PON1 (192) (Q/Q vs (Q/R vs R/R)), and PON2 (311) (S/S vs (S/C vs C/C) to obtain groups of similar size. We assessed heterozygosity vs homozygosity for each SOD2 intronic polymorphism. For the A16V SOD2 polymorphism, we combined the V/V vs (AA vs AV) risk genotypes.

We used logistic regression analysis to compare the NIHL group vs the control group for biochemical indices, age, smoking, and genotypes and calculated the odds ratios (ORs) and the 95% confidence intervals (95% CIs). To test whether the 0% non-/light smokers in the control group affected the data, we removed smokers from the NIHL group and compared the genotypes in the two groups (controls and NIHL, both non-/light smokers) by logistic regression analysis. We then forced the statistics by including a fictitious smoker among the controls and again compared the genotypes and other variables in the

**Table 1. PCR and DHPLC conditions for SOD2 gene screening.**

<table>
<thead>
<tr>
<th>Exon</th>
<th>Forward</th>
<th>Reverse</th>
<th>Annealing temperature, °C</th>
<th>Product size, bp</th>
<th>DHPLC temperatures, °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5'-TTGGCAACGCCCTTCAG-3'</td>
<td>5'-TGCAAGCCACTGTGGCCATTG-3'</td>
<td>62</td>
<td>167</td>
<td>65, 66, and 67</td>
</tr>
<tr>
<td>2</td>
<td>5'-CAGCCGACGGCCTTCAG-3'*'</td>
<td>5'-TATGGGCGCTGGCTCCCTCCT-3'</td>
<td>65</td>
<td>335</td>
<td>64 and 65</td>
</tr>
<tr>
<td>3</td>
<td>5'-CTGGTCAATTATAGGGGCAG-3'</td>
<td>5'-ATCACGAAACCAGGAAG-3'</td>
<td>53</td>
<td>418</td>
<td>57 and 58</td>
</tr>
<tr>
<td>4</td>
<td>5'-TTTTAATTATTGGGGGC-3'</td>
<td>5'-AAAGCTCTGGTGTTATCTGGAAG-3'</td>
<td>52</td>
<td>434</td>
<td>56 and 57</td>
</tr>
<tr>
<td>5</td>
<td>5'-AGGTTAAGAGGAGATGC-3'</td>
<td>5'-GCTAACATACCTCAGTATACG-3'</td>
<td>50</td>
<td>250</td>
<td>56, 57, and 58</td>
</tr>
</tbody>
</table>

*As described elsewhere (8).*
Results

There were no statistically significant differences between NIHL and control individuals regarding biochemical findings (Table 2), although total cholesterol, HDL, and LDL were slightly higher in NIHL individuals than in controls. However, the NIHL group was older ($P < 0.001$) and included a significantly higher percentage of smokers ($P < 0.001$).

The genotype distributions of the $PON1$ M55L and Q192R and $PON2$ S311C polymorphisms in the whole cohort and in the NIHL and control individuals are shown in Table 3. The relative frequencies of the $PON1$ Q192R polymorphism did not differ significantly between NIHL individuals and controls, whereas the $PON1$ (55) L allele was more frequent in NIHL individuals than in controls ($P = 0.005$), and $PON2$ (311) (C/C) was present only in NIHL individuals.

The screening of the $SOD2$ gene by DHPLC revealed three known polymorphisms (A16V, IVS3-23T/G, and IVS3-60T/G) and two novel polymorphisms (IVS1+8A/G and IVS3+107T/A). Their relative frequencies did not differ between NIHL and control individuals (Table 4). The genotype distributions of the $PON1$, $PON2$, and $SOD2$ polymorphisms evaluated in the study cohort ($n = 94$) and in individuals with normal hearing ($n = 31$) were in Hardy–Weinberg equilibrium. Despite a significant linkage disequilibrium ($P = 0.003$), which favored the simultaneous presence of the R and L alleles, there was no statistically significant association between the different $PON1$ 55/192 haplotypes and NIHL, except for the MMQQ haplotype, which was not found in NIHL individuals ($P = 0.001$). Concerning $SOD2$, linkage disequilibrium was found between $SOD2$ IVS1+8A/G and $SOD2$ A16V ($P = 0.003$); $SOD2$ A16V and $SOD2$ IVS3+107T/A ($P < 0.001$); $SOD2$ IVS3+107T/A and $SOD2$ IVS3-23T/G, IVS3-60T/G ($P < 0.001$); and $SOD2$ A16V and $SOD2$ IVS3-23T/G, IVS3-60T/G ($P < 0.001$), which favored the simultaneous presence of A alleles of both $SOD2$ IVS1+8/$SOD2$ 16 and $SOD2$ 16/$SOD2$ IVS3+107, and the A allele of $SOD2$ IVS3+107 with the G allele of $SOD2$ IVS3-23/$SOD2$ IVS3-60. However, there were no significant associations between the different $SOD2$ haplotypes and NIHL.

ANOVA did not reveal any significant associations between biochemical markers and $PON1$, $PON2$, or $SOD2$ polymorphisms in either NIHL individuals or controls (data not shown). To eliminate the effects of age and

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Total cohort</th>
<th>Individuals with normal hearing</th>
<th>NIHL individuals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>Mean (SD)</td>
<td>43 (7)</td>
<td>39 (5)</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>Mean (SD)</td>
<td>5.04 (1.00)</td>
<td>4.90 (1.07)</td>
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<tr>
<td>Urea (mmol/L)</td>
<td>Mean (SD)</td>
<td>6.35 (1.30)</td>
<td>6.42 (1.42)</td>
</tr>
<tr>
<td>Creatinine (mmol/L)</td>
<td>Mean (SD)</td>
<td>76.98 (8.15)</td>
<td>77.48 (7.65)</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>Mean (SD)</td>
<td>5.08 (1.00)</td>
<td>4.82 (0.99)</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/L)</td>
<td>Mean (SD)</td>
<td>1.02 (0.22)</td>
<td>0.98 (0.20)</td>
</tr>
<tr>
<td>LDL-cholesterol (mmol/L)</td>
<td>Mean (SD)</td>
<td>3.25 (0.87)</td>
<td>3.06 (0.78)</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>Mean (SD)</td>
<td>1.78 (0.89)</td>
<td>1.71 (1.03)</td>
</tr>
<tr>
<td>TRAP (mmol/L)</td>
<td>Mean (SD)</td>
<td>1.29 (0.05)</td>
<td>1.29 (0.05)</td>
</tr>
<tr>
<td>Smokers (&gt;10 cigarettes/day), %</td>
<td>34</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Non-/light smokers (≤10 cigarettes/day), %</td>
<td>69</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

*All participants were exposed to a mean (SD) noise level of ~92.4 (4.1) dB (A) for 20 years.

$P < 0.001$ vs controls by t-test.

$P < 0.001$ vs controls by χ² test.
Here we show that adjustment for these confounding factors, the association smoking, we tested by logistic regression analysis, after the oxidative state of cells induced by hydrogen peroxide in the stria vascularis (1, 29, 30). Thus, also in humans, Corti’s ciliated cells might be damaged by local release of free radicals, which in turn may lead to a neurosensorial hearing loss, particularly in individuals bearing the mitochondrial reactive oxygen species (32) and therefore, the SOD2 enzyme protects against damage caused by free radicals (19). The ubiquitously produced PON2 acts as an antioxidant enzyme; thus, its overproduction is capable of lowering the oxidative state of cells induced by hydrogen peroxide.

Our data showing that the PON2 C allele is associated with NIHL (OR = 5.01; 95% CI, 1.11–22.54) suggest a genetic predisposition to this disorder. The pathogenesis of NIHL may involve the release of oxygen species consequent to chronic exposure to high sound levels that may damage Corti’s organ. In fact, exposure to noise in animal models appears to increase the concentrations of superoxide radicals in the cochlear fluid as well as in the stria vascularis (1, 29, 30). Thus, also in humans, Corti’s ciliated cells might be damaged by local release of free radicals, which in turn may lead to a neurosensorial hearing loss, particularly in individuals bearing the PON2 C allele. Our data are in agreement with the association between the PON2 S311C polymorphism and Alzheimer dementia, a neurodegenerative disease in which oxidative stress may play an important role (23). Concerning PON1, a HDL-associated enzyme produced mainly in the liver that plays a major role in such diseases as atherosclerosis (12, 13), we did not detect any significant association between the PON1 Q192R polymorphism and NIHL, and the significant increase of the SOD2 enzyme protects against damage caused by free radicals (19).

In the rat cochlear labyrinth, the SOD2 enzyme protects against damage caused by free radicals (31). Furthermore, SOD2-knockout mice have enhanced susceptibility to alterations caused by other mitochondrial enzymes and to diseases resulting from increased concentrations of mitochondrial reactive oxygen species (32). We detected three known (A16V, IVS3-23T/G, and IVS3-60T/G) and two novel (IVS1+8A/G and IVS3+107T/A) polymorphisms

<table>
<thead>
<tr>
<th>Genotype, n (%)</th>
<th>Total cohort</th>
<th>Individuals with normal hearing</th>
<th>NIHL individuals</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/A</td>
<td>50 (54)</td>
<td>14 (47)</td>
<td>36 (57)</td>
</tr>
<tr>
<td>G/G</td>
<td>7 (7)</td>
<td>1 (3)</td>
<td>6 (10)</td>
</tr>
<tr>
<td>G/A</td>
<td>36 (39)</td>
<td>15 (50)</td>
<td>21 (33)</td>
</tr>
<tr>
<td>P</td>
<td>0.234</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 4. Genotype distributions of SOD2 IVS1+8A/G, A16V, IVS3+107T/A, IVS3-23T/G, and IVS3-60T/G polymorphisms in the enrolled cohort.**

**Discussion**

Here we show that PON2 (SC+CC) genotypes and the IVS3-23T/G, IVS3-60T/G SOD2 polymorphisms are associated with NIHL irrespective of age and smoking habits. The ubiquitously produced PON2 acts as an antioxidant enzyme; thus, its overproduction is capable of lowering the oxidative state of cells induced by hydrogen peroxide (19).
in the SOD2 gene in our cohort. The frequencies of SOD2 A16V genotypes matched those reported for other Caucasian populations (6) and did not differ between NIHL and control individuals. Similarly, this polymorphism is unrelated to degenerative diseases such as Parkinson disease (9, 33) and amyotrophic lateral sclerosis (8). In contrast, the SOD2 A16A genotype is associated with increased breast cancer risk (6), a high degree of carotid atherosclerosis (7), and to exudative age-related macular degeneration in Japanese individuals, in whom allele A occurs less frequently than in Caucasians (34).

Finally, although it has been reported that A16A homozygotes may have higher SOD2 activity than V16V homozygotes (35), in our cohort the number of individuals bearing the A allele did not differ between NIHL and control individuals. Thus, this polymorphism does not appear to influence susceptibility to noise-induced damage.

The heterozygous frequency of the two novel polymorphisms IVS1+8A/G and IVS3+107T/A did not differ between NIHL and control individuals; however, these polymorphisms could provide a tool for investigating other SOD2-related diseases and for linkage disequilibrium mapping. SOD2 polymorphisms IVS3-23T/G and IVS3-60T/G showed a high heterozygosity, and their genotype frequencies were similar to those reported previously (8). They were clearly associated with NIHL (OR = 5.09; 95% CI, 1.27–20.47); however, given their initial localization, it is unlikely that they are involved in the development of NIHL and they may function, instead, as markers that are in linkage disequilibrium with other polymorphisms.

Concerning the effect of smoking on NIHL, it has been reported that smokers have a greater risk of hearing loss than nonsmokers (36, 37). Recently, Palmer et al. (38) reported an additive rather than a multiplicative interaction between smoking and noise exposure. They concluded that although workers exposed to long-term noise should be discouraged from smoking, the extra risk of smoking on hearing loss, in environments where noise levels are significant, is small relative to that of noise itself.

In conclusion, the association of PON2 and SOD2 polymorphisms with neurosensorial hearing could represent a marker of susceptibility to NIHL independent of the smoking effect, although the risk associated with smoking was surprisingly large in our study.

This work was supported by grants from Ministero del Lavoro, Regione Campania and MIUR Cluster 04. We thank Laura Tudisco for technical assistance. We are grateful to Jean Gilder for editing the text.

References


