

NF- κ B Pathway Protects Cochlear Hair Cells From Aminoglycoside-Induced Ototoxicity

Hongyan Jiang, Su-Hua Sha, and Jochen Schacht*

Kresge Hearing Research Institute, Department of Otolaryngology, University of Michigan, Ann Arbor, Michigan

Cell death in outer hair cells of the mammalian inner ear induced by aminoglycoside antibiotics is mediated by reactive oxygen species (ROS) and can be prevented by antioxidants. The current study investigates the role of the nuclear factor (NF)- κ B pathway in cell death or survival in adult CBA mice. Kanamycin (700 mg/kg subcutaneously, twice per day) progressively destroys hair cells but after 7 days of treatment auditory function and morphology are not yet affected significantly, permitting investigations of early events in drug-induced cell death. Immunostaining for 4-hydroxynonenal, indicative of lipid peroxidation, was elevated in the cochlea, but there was no effect on nitrotyrosine, a marker for peroxynitrite. NF- κ B was increased at 3 hr, 3 days, and 7 days of treatment, with p50 and p65 proteins as its most abundant subunits. Immunoreactivity for p50 was present in nuclei of inner hair cells and supporting cells that survive the drug treatment. In contrast, nuclei of outer hair cells were devoid of label. Concomitant injections of antioxidants, however, such as 2,3-dihydroxybenzoic acid or salicylate (which prevent cell death induced by kanamycin), promoted the translocation of NF- κ B into the nuclei of outer hair cells. In addition, kanamycin treatment decreased tyrosine phosphorylation of the inhibitory I κ B α protein, leading to increased I κ B α levels in the cochlea; the effect was reversed by cotreatment with antioxidants. These results suggest that changes in the redox state of the cochlea stimulate the activation of NF- κ B and that this activation is cell protective. © 2005 Wiley-Liss, Inc.

Key words: kanamycin; reactive oxygen species; lipid peroxidation; redox signaling, I κ B

A variety of external stimuli, such as excessive noise and certain medications, as well as the aging process can damage sensory cells of the auditory organ, presumably by their ability to cause oxidative stress. Because neither inner nor outer hair cells of the cochlea regenerate, the ensuing sensorineural hearing loss is permanent. The regulation of cell death and survival in response to noxious stimuli is therefore a crucial aspect of inner ear homeostasis and function.

Oxidant stress has been implicated as a causative factor in aminoglycoside-induced hearing loss or “ototoxicity,” a side effect of therapy with these commonly used drugs (Forge and Schacht, 2000). Formation of reactive

oxygen species (ROS) by aminoglycosides is supported by *in vitro* evidence for the existence of redox-active gentamicin-iron-lipid complexes (Priuska and Schacht, 1995; Sha and Schacht, 1999a) and by *in vivo* studies showing protection against ototoxicity by antioxidants in guinea pigs and mice (Song and Schacht, 1996; Sha and Schacht 1999b; Wu et al., 2002). Furthermore, mice overexpressing superoxide dismutase were protected against kanamycin-induced hearing loss (Sha et al., 2001b).

Cellular responses to a traumatic insult include activation of multiple signaling pathways that affect gene expression, leading either to cell survival by restoring homeostasis, or to cell death via apoptotic or necrotic pathways. The ability of ROS to signal cell survival or death seems to be linked to modulation of gene expression through activation or inhibition of redox-sensitive transcription factors. Nuclear factor (NF)- κ B in particular has been implicated in ROS signaling (Bonizzi et al., 1999; Bowie and O’Neill, 2000; Dröge, 2002). NF- κ B is a heterodimer of two protein subunits and resides in the cytoplasm in an inactive form bound to the inhibitory protein I κ B. Various stimuli initiate a cascade of events that leads to the phosphorylation, ubiquitination, and subsequent degradation of the inhibitory I κ B protein, thus activating and translocating NF- κ B to the nucleus.

The downstream events triggered by a transcription factor such as NF- κ B can be rather divergent, and the ultimate fate of a cell may depend on the stimulus and the cell type involved (Bonizzi et al., 2000). Aside from being the requisite tissue for investigations into ototoxic mechanisms, the peripheral auditory system provides several unique advantages for research into the relationships between metabolism, structure and activity. The cochlear neuroepithelium consists of both sensory and nonsensory

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*Correspondence to: Dr. Jochen Schacht, Kresge Hearing Research Institute, 1301 East Ann Street, Ann Arbor MI 48109-0506.

E-mail: schacht@umich.edu

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cells, with distinctly different sensitivity to various well-defined traumata such as drugs with a preferential toxicity to the auditory system (e.g., aminoglycoside antibiotics) and excessive noise exposure. The effects of these ototoxic insults can be assessed quantitatively by physiologic measurements in vivo and morphologic assessments postmortem. In vivo assessments allow for minimally invasive longitudinal studies that can distinguish the function of the outer hair cells or the inner hair cell synapse. Postmortem analysis of pathology in the organ of Corti is highly simplified by the fact that lost inner hair cells and outer hair cells are replaced in an orderly process by scar tissue. A surface preparation of the organ of Corti clearly outlines existing hair cells and the spaces where hair cells are missing, allowing for a quantitative assessment of pathologic changes. Furthermore, immunocytochemistry on cross sections of the cochlea can simultaneously distinguish events in the two types of sensory cells and the nonsensory accessory structures.

This study examines activation of NF- κ B and its relation to cell death or survival in the inner ear of CBA mice treated with the ototoxic aminoglycoside kanamycin. In addition, we analyze the effect of combined treatment with antioxidants (which prevents the morphologic and functional damage induced by kanamycin) on activation of NF- κ B and I κ B proteins.

MATERIALS AND METHODS

Materials

Kanamycin sulfate was purchased from USB Corporation (#17924; Lot 110755; Cleveland, OH); [γ -³²P]ATP from NEN Life Science Products Inc. (Boston, MA), T4 polynucleotide kinase and oligonucleotides with a base length of 21mer containing the consensus core sequence for NF- κ B (5'-AGT TGA GGG GAC TTT CCC AGG C-3') were obtained from Promega (Madison, WI). Poly(dIdC), MicroSpin G-50 columns, and enhanced chemiluminescence (ECL) chemicals as Western blotting detection reagents were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Molecular weight standards (BenchMark Protein ladders) were purchased from Invitrogen Life Technologies (Carlsbad, CA), antibodies for immunochemistry of 4-hydroxynonenal (4-HNE) and nitrotyrosine were from Calbiochem (San Diego, CA). NF- κ B subunits including rel-B, c-Rel, p50, p65, p52 and I κ B α , I κ B β as well as I κ B α -agarose conjugate for immunoprecipitation came from Santa Cruz Biotechnology Inc. (Santa Cruz, CA), PY-plus mouse anti-phosphotyrosine (cocktail) was obtained from Zymed Laboratories Inc. (San Francisco, CA), and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was from Chemicon International (Temecula, CA). Secondary antibodies for Western blotting were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA), and secondary fluorescent antibodies (Alexa 488 and Alexa 546), rhodamine phalloidin, propidium iodide, and Hoechst 33342 were from Molecular Probes Inc. (Eugene, OR). Complete Mini EDTA-free protease inhibitor cocktail tablets were from Roche Diagnostic GmbH (Mannheim, Germany). SV Total RNA Isolation Kit was purchased from Promega Corporation

(Madison, WI), and SuperScript First-Strand Synthesis Kit from Invitrogen Corporation (Carlsbad, CA). Primers for the sequences of I κ B α and GAPDH were obtained from Integrated DNA Technology (Coralville, IA). All other reagents came from Sigma (St. Louis, MO).

Animals and Drug Administration

Male CBA mice were received at an age of 4 weeks from Harlan Sprague-Dawley Co. (Indianapolis, IN). The animals had free access to water and a regular mouse diet (Purina 5025, St. Louis, MO) and were acclimated for 1 week before experimental procedures were begun. All experimental protocols were approved by the University of Michigan Committee on Use and Care of Animals. Animal care was under the supervision of the University of Michigan's Unit for Laboratory Animal Medicine.

For most experiments, mice were separated into four groups that received twice-daily subcutaneous injections for 7 days of, respectively: (1) saline; (2) sodium salicylate (150 mg/kg body weight in saline); (3) kanamycin (700 mg of kanamycin base/kg body weight); or (4) kanamycin plus salicylate or kanamycin plus 2,3-dihydroxybenzoate (DHB; 300 mg/kg body weight in 3% sodium bicarbonate) in separate injections but at the same time. Animals were sacrificed and the cochleae were collected 1, 3, and 6 hr after the first injection on Day 1, or 3 hr after the second injection on Day 3, 7, and 14.

Evaluation of Auditory Function and Surface Preparations

Auditory thresholds were measured by evoked auditory brainstem responses, and histopathology of the hair cells was assessed by surface preparations. To study the long-term effects of drug treatment, thresholds were taken for each animal before the beginning of the study, 7 days and 2 weeks after the start of drug treatment, and then after 3 and 5 weeks. Auditory brainstem response measurements and surface preparations were carried out as described previously (Wu et al., 2001).

Extraction of Total Protein

The cochleae were removed rapidly and were dissected in ice-cold 10 mM phosphate-buffered saline (PBS). Tissue from one mouse cochlea was homogenized in ice-cold RIPA buffer (#20-188; Upstate, Waltham, MA) using a micro Tissue Grind pestle (Kontes Glass Company, NJ) for 30 sec. For immunoprecipitation assays, phosphatase inhibitor cocktail I and II (Sigma) at 10 μ l/ml each were added to the RIPA buffer. The homogenates were kept on ice for 15 min, and then centrifuged at 15,000 \times g at 4°C for 10 min.

Extraction of Nuclear and Cytosolic Proteins

Cochleae were removed rapidly and dissected in 10 mM ice-cold PBS. Tissues from three mice were pooled and homogenized in cytoplasmic lysis buffer (10 mM sodium HEPES, pH 7.9, additionally containing 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 5 mM dithiothreitol [DTT], 10 mM each of the phosphatase inhibitors NaF and sodium β -glycerophosphate, 1 μ g/ml [*p*-amidinophenyl] methanesulfonyl fluoride, and 1/10 tablet/ml of Complete Mini EDTA-free protease inhibitor cocktail) by using a micro Tissue Grind pestle for 10 sec. The

homogenates were kept on ice for 15 min and then centrifuged at $500 \times g$ at 4°C for 10 min to obtain a crude nuclear pellet and a first supernatant. The supernatant was centrifuged at $15,000 \times g$ for 10 min at 4°C , and the second supernatant was collected as cytosolic protein. The crude nuclear pellet was washed twice with a cytosolic lysis buffer and centrifuged each time at $15,000 \times g$ at 4°C for 5 min. The washed nuclear pellets were resuspended in a nuclear lysis buffer {50 mM Tris-HCl, pH 7.5, containing 10% glycerol, 400 mM KCl, 1 mM EDTA, 1 mM EGTA, 5 mM DTT, and the aforementioned phosphatase and protease inhibitors}, and kept on ice for 30 min. The suspensions were centrifuged at $15,000 \times g$ at 4°C for 10 min, and the supernatant was collected as the nuclear protein extract. Both cytosolic protein and nuclear extract were stored at -80°C until analyzed. Protein concentrations were measured by the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA).

Electrophoretic Mobility Shift and Supershift Assays

Double-stranded oligonucleotides with a base length of 21mer containing the consensus core sequence for NF- κB (5'-AGT TGA GGG GAC TTT CCC AGG C-3') were end labeled with [γ - ^{32}P]ATP by T4 polynucleotide kinase and the unincorporated nucleotides were removed from the probe with a MicroSpin G-50 column. Nuclear or cytosolic proteins (10 μg) were incubated for 10 min at 25°C with a binding buffer (final concentrations, 50 mM Tris-HCl, pH 7.5, 0.25 $\mu\text{g}/\text{ml}$ of poly[dIdC], 20% glycerol, 5 mM MgCl_2 , 2.5 mM EDTA, 2.5 mM DTT, and 250 mM NaCl). Labeled probes (0.5–5 $\times 10^6$ cpm/pmol) were then added and the incubation continued for 30 min in a total volume of 20 μl . For supershift assays, cochlear homogenates (10 μg) were incubated with antibodies (4 μg) against the individual NF- κB subunit proteins p65, p50, p52, rel-B, and c-Rel at 25°C for 1 hr before the labeled probe was added. The reaction mixture was electrophoresed on a 4.5% nondenaturing polyacrylamide gel in a running buffer containing 50 mM Tris, 0.38 M glycine and 2 mM EDTA, final pH 8.5, at a constant voltage of 11 V/cm for about 1.5 hr. Gels were vacuum dried with a Slab Gel Dryer (Savant Instruments Inc., Holbrook, NY) at 70°C for 45–50 min, the dried gels were exposed to a Phospho Imager screen overnight, and results were visualized with a Typhoon 9400 (Amersham).

RNA Isolation and RT-PCR

Total RNA was isolated from whole cochleae of mice (five mice pooled per assay) according to the instructions for the SV Total RNA Isolation System. For RT-PCR, single-stranded cDNA was prepared from 2 μg of total RNA by using reverse transcriptase with an oligo-dT primer (Invitrogen). Two μl of each sample of cDNA were subjected to PCR amplification using specific primers. The primers for I $\kappa\text{B}\alpha$ were the 299-base forward primer 5'-CAT GAA GAG AAG ACA CTG ACC ATG GAA-3' and the 627-base reverse primer 5'-TGG ATA GAG GCT AAG TGT AGA CAC G-3', which yield a 328-base pair (bp) product. The primers for GAPDH detection were the 796-base forward primer 5'-GCC AAG TAT GAT GAC ATC AAG AAG and the 1,059-base reverse primer 5'-TCC AGG GGT TTC TTA CTC CTT GGA, which yield a 264-bp product (Gavrilyuk et al., 2002). PCR was initiated by a hot-start method, and conditions set to 35 cycles of denaturation at

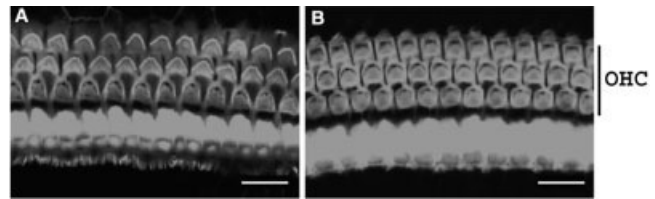


Fig. 1. Kanamycin treatment for 7 days does not alter morphology of the organ of Corti. Surface preparations were obtained and stained for actin with rhodamine phalloidin. Sections were taken from the basal turn of the cochlea and are representative of three animals examined under each condition. Scale bar = 10 μm . **A:** Preparation from a saline-treated animal (for 7 days) showing the regular outline of three rows of outer hair cells (OHC). **B:** Preparation from an animal treated with kanamycin for 7 days showing almost normal morphology.

94°C for 20 sec, annealing at 60°C for 25 sec, and extension at 72°C for 30 sec, followed by 3 min at 72°C . PCR products were separated by electrophoresis on 2% agarose gels containing 0.1 $\mu\text{g}/\text{ml}$ ethidium bromide and visualized under UV light using a MultiImage Light Cabinet (Alpha Innotech Corp., San Leandro, CA).

Western Blot Analysis

The protein samples (50 μg) were separated on 10% SDS-PAGE gels. After electrophoresis, the proteins were transferred onto polyvinylidene difluoride (PVDF) membrane (Amersham), and blocked with 5% nonfat dry milk in PBS-0.1% Tween 20 (PBS-T). The membranes were incubated with anti-I $\kappa\text{B}\alpha$ (1:2,000) or anti-I $\kappa\text{B}\beta$ (1:250) rabbit polyclonal antibody for 2 hr, and washed three times with PBS-T buffer (10 min each time). Membranes then were incubated with a secondary antibody (goat anti-rabbit IgG), coupled to horseradish peroxidase at a concentration of 1:10,000 for 1 hr. After extensive washing of the membranes, immunoreactive bands were visualized by ECL according to the instructions of the manufacturer (Amersham). The membranes were then stripped and restained for anti-GAPDH at a concentration of 1:20,000 as a control for sample loading.

Immunoprecipitation

Agarose-conjugated anti-I $\kappa\text{B}\alpha$ antibody (2 μg) was mixed with 100 μg of total cochlear protein in an Eppendorf tube (minimum of 300 μl /per tube). It was rocked overnight at 4°C , centrifuged at $1,000 \times g$ at 4°C for 3 min, and the supernatant was removed. The pellets were washed three times with the lysis buffer and after the final wash, the pellets were resuspended in 40 μl of $2\times$ electrophoresis sample buffer (including 0.5 M Tris-HCl, 4% SDS, 20% glycerol, 0.02% bromophenol blue, and 10% β -mercaptoethanol). Subsequently, mouse anti-phosphotyrosine (1:200) was detected by using Western blotting.

Immunocytochemistry

Mice were decapitated and the temporal bones were removed quickly. Cochleae were fixed immediately by slowly perfusing cold 4% paraformaldehyde from a syringe through the round window to an exit hole created in the apex. Cochleae

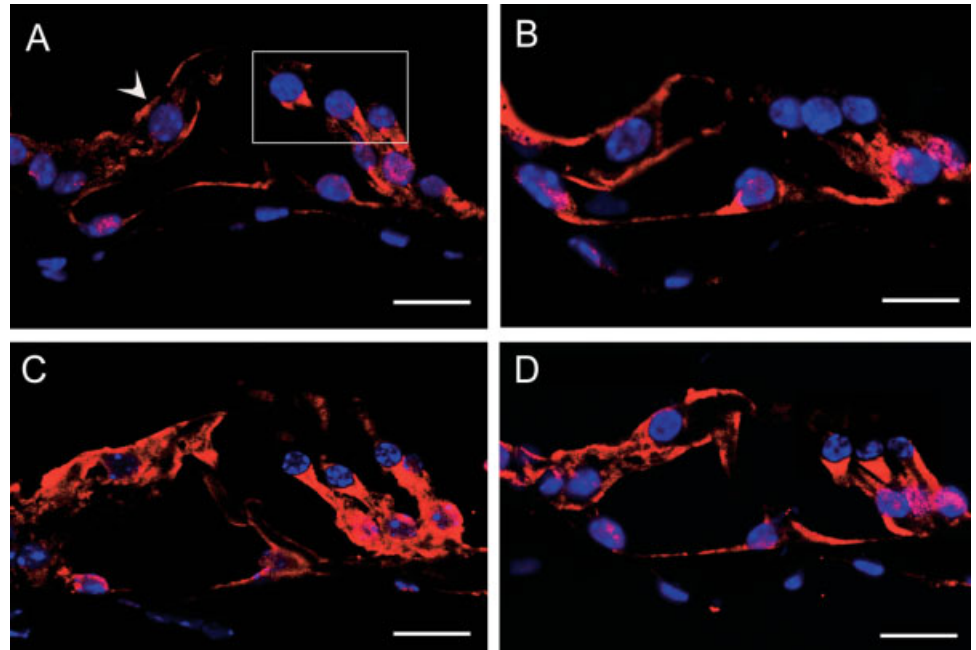


Fig. 2. Staining for 4-HNE in the cochlea. Cryosections of the cochlea, showing inner hair cells (arrowhead), three rows of outer hair cells (rectangle) and supporting cells, were stained with anti-4-HNE antibody (red); blue fluorescence is Hoechst 33342, indicating nuclei. Sections shown are representative of three mice in each group. Scale bar = 10 μ m. Saline-treated controls (A) and animals injected with salicylate (B) showed weak staining for 4-HNE throughout the cochlea. Immunostaining increased after kanamycin treatment for 7 days (C) and was attenuated by cotreatment with salicylate (D).

remained in 4% paraformaldehyde overnight at 4° C. Cryostat sections of 5 μ m were incubated in 0.5% Triton X-100 for 15 min at room temperature. The sections were then washed three times with PBS and blocked with 10% goat serum for 30 min at room temperature, followed by incubation with the primary antibody at a concentration of 1:100 (anti-4HNE), 1:200 (anti-nitrotyrosine), or 1:1,000 (anti-p50 and c-Rel) at 4°C for 72 hr. The sections were then incubated with the secondary antibody (Alexa 488 or Alexa 546 conjugated) at a concentration of 1:500 at 4°C overnight in darkness. After washing three times with PBS, the sections for detection of 4-HNE and nitrotyrosine staining were incubated with Hoechst 33342 (2 μ g/ml in PBS) at room temperature for 40 min. After a final wash with PBS, the slides were mounted. Control incubations were routinely processed without primary antibody. Immunolabeling was visualized and imaged using a Zeiss laser confocal microscope.

Statistical Analysis

Results reported are generally representative of at least three independent replications, as indicated in the legends to the figures. When appropriate, data were evaluated statistically by Student's *t*-test and by analysis of variance (ANOVA) with Student-Newman-Keuls test for significance ($P < 0.05$) using Primer of Biostatistics software (McGraw-Hill Software, New York, NY).

RESULTS

Mice Maintain Normal Cochlear Morphology and Function After 7-Day Kanamycin Treatment

Our earlier work had established an adult mouse model of aminoglycoside-induced hearing loss (Wu et al., 2001). Injections with 700 mg kanamycin base/kg body weight twice per day for 14 days will lead to severe loss of

hair cells and auditory function, which can be prevented by concurrent treatment with DHB, 2-hydroxybenzoic acid (salicylate), or other antioxidants. In contrast, a modified drug regimen of 7-day dosing only, used in the present study, did not significantly affect auditory morphology or function. Surface preparations of the organ of Corti revealed intact hair cells after kanamycin treatment for 7 days (Fig. 1). Auditory thresholds showed only a minor elevation, from 20 ± 4 dB before injections to 28 ± 6 dB after 7 days (24 kHz; mean \pm SD; $n = 5$). Furthermore, neither DHB or salicylate injections alone nor the combination of kanamycin with DHB or salicylate affected thresholds or morphology. Throughout this study, there were no changes in any parameters tested between untreated control mice and saline-injected animals.

Oxidative Stress in Kanamycin-Treated Animals

Immunostaining for the lipid peroxidation marker 4-HNE increased after kanamycin treatment (Fig. 2), corroborating the notion that aminoglycoside antibiotics produce ROS *in vivo*. Increased immunostaining was seen in all cell types, and cotreatment with DHB or salicylate reduced the overall levels of 4-HNE. In contrast, staining for nitrotyrosine, a marker for the action of peroxynitrite, remained unaffected by the drug treatment (Fig. 3).

Kanamycin Increases NF- κ B Binding Activity in Nuclear and Cytosolic Extracts of the Cochlea

Electrophoretic mobility shift assays of nuclear extracts with a radiolabeled oligonucleotide probe showed a band corresponding to NF- κ B whose intensity remained unchanged during 14 days of saline injections (Fig. 4A). In contrast, NF- κ B increased 3 hr after the first injection of

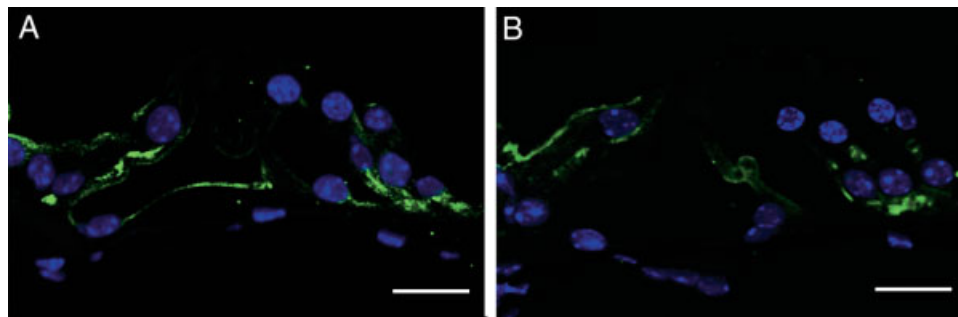


Fig. 3. Staining for nitrotyrosine in the cochlea. Cryosections of the cochlea were stained with anti-nitrotyrosine antibody (green); blue fluorescence is Hoechst 33342, indicating nuclei. Staining for nitrotyrosine was not changed by treatment with kanamycin (**A**, control, vs. **B**, kanamycin). Sections are representative of three mice in each group. Scale bar = 10 μ m.

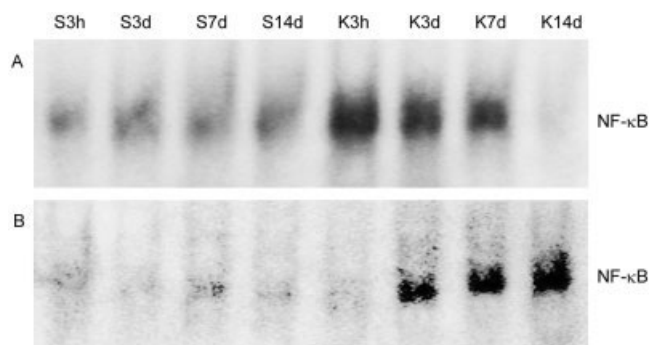


Fig. 4. Kanamycin treatment increases NF- κ B. Proteins from cochlear nuclear extracts or cytosolic supernatants bound to a radiolabeled double-stranded oligonucleotide probe with a recognition sequence for NF- κ B in a gel shift assay. **A**: Constitutive expression of NF- κ B is found in nuclear extracts of saline-treated control animals (S) independent of the duration of saline treatment. The signal for NF- κ B was increased significantly 3 hr after a single kanamycin injection (K) and 3 and 7 days after twice-daily kanamycin treatment. **B**: In cytosolic extracts, NF- κ B was barely detectable in saline control animals (S) but was increased significantly after 3, 7 and 14 days of twice-daily kanamycin treatment. S3h, S3d, S7d, S14d indicate samples from saline-injected control animals at 3 hr, 3 days, 7 days and 14 days, respectively; K3h, K3d, K7d and K14d indicate samples from kanamycin-treated animals at the same time points. All animals were sacrificed 3 hr after the last injection. A is a representative example from five individual assays; B is one of two individual assays.

kanamycin. The intensity of the band remained elevated after 3 and 7 days of treatment but was reduced after a 14-day treatment. No band was detected in the absence of a nuclear extract (not shown). In cytosolic extracts (Fig. 4B), a NF- κ B signal appeared after 3 days of kanamycin injections and persisted at 7 and 14 days. Based on the electrophysiologic and morphologic data and this time course, we chose a 7-day treatment as a basis for subsequent detailed biochemical and molecular biological analyses.

P50 and p65 Are Two Abundant Subunits of NF- κ B

Cochlear extracts from animals treated for 7 days were subjected to supershift assays for the analysis of NF- κ B composition (Fig. 5). After incubation with antibodies to individual NF- κ B subunit proteins, the bands for

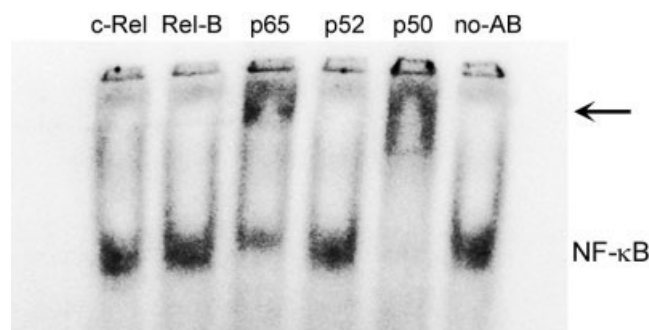


Fig. 5. P50 and p65 are the major subunits of NF- κ B. After 7 days of treatment with kanamycin, cochlear homogenates were incubated individually with the protein subunits of NF- κ B, indicated as c-Rel, Rel-B, p65, p52, and p50, and subjected to an electromobility shift assay. No-AB designates incubation without antibody. P50 and p65 antibodies induced major supershifts of the radioactive band to a more slowly migrating position on the gel (arrow). The figure is representative of four independent experiments.

p50 and p65 showed a major supershift to a more slowly migrating position on the gel. Other antibodies had a lesser effect on the mobility of the NF- κ B band. These supershifts induced by specific antibodies indicate that the p50 and p65 proteins are major components of the NF- κ B dimer.

Cotreatment With Salicylate Promotes NF- κ B Translocation Into Outer Hair Cell Nuclei

In cryosections of the cochlea, weak immunostaining for p50 was seen in the cytosol and some nuclei (inner hair cells; supporting cells) of saline-treated animals (Fig. 6A). After 7 days of kanamycin treatment (Fig. 6D), p50 staining increased strongly in the cytosol of all cell types. A nuclear localization was only seen in inner hair cells and supporting cells, however, whereas nuclei of outer hair cells remained devoid of staining (Fig. 6D, inset). Treatment with DHB or salicylate alone did not induce major changes (Fig. 6B,C) but concurrent treatment with kanamycin plus DHB or salicylate increased p50 staining in the nuclei of outer hair cells (Fig. 6E,F, inset). RT-PCR analysis for p105, the precursor of p50, showed similar levels of mRNA under all experimental conditions (not shown).

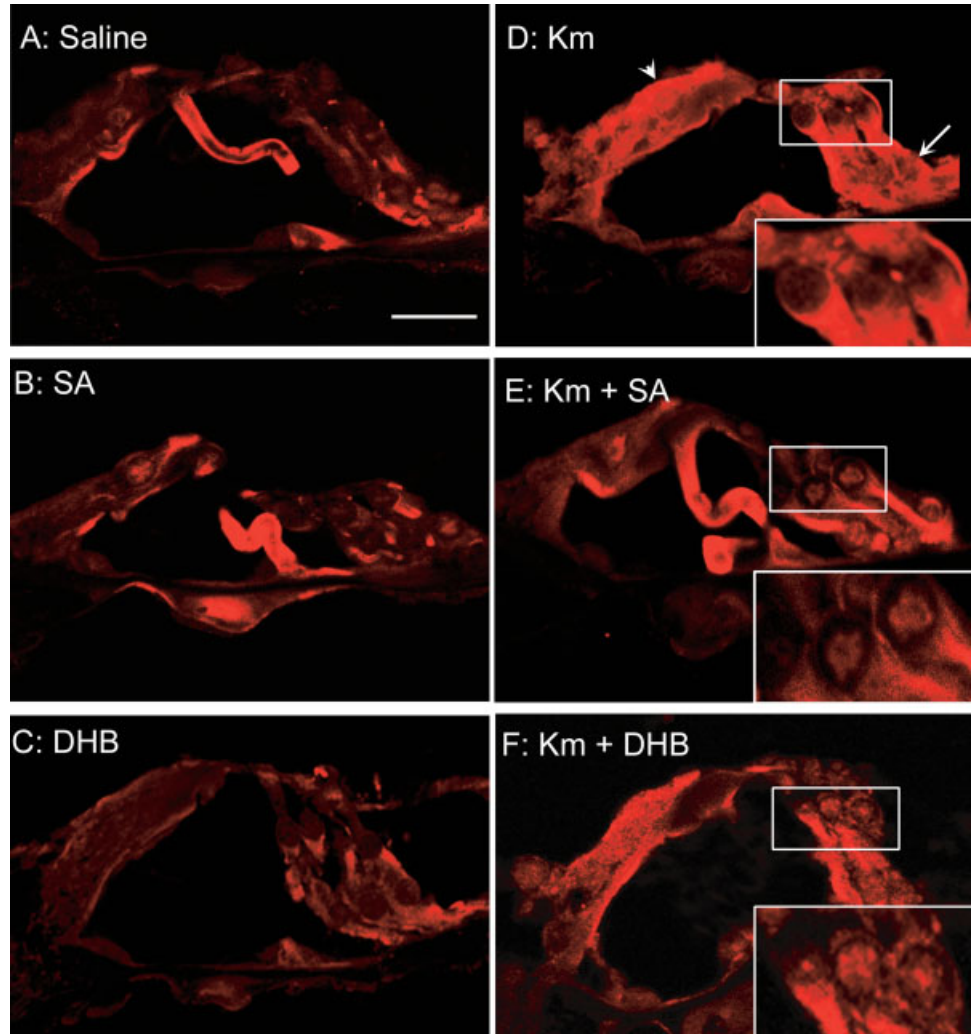


Fig. 6. Antioxidant therapy promotes the nuclear translocation of NF- κ B in outer hair cells. Immunocytochemistry against the p50 protein was carried out on tissue sections after treatment of animals with saline (A), salicylate alone (B), DHB alone (C), kanamycin (D), kanamycin plus salicylate (E) or kanamycin plus DHB (F). Kanamycin treatment increased cytosolic staining in all cell types but nuclear staining only in inner hair cells (arrowhead) and supporting cells (arrow). Nuclei of outer hair cells (D, and insert of higher magnification) remained unstained. Cotreatment with salicylate (E) or DHB (F) induced anti-p50 immunoreactivity in outer hair cell nuclei (frame and insert at 2 \times magnification). Salicylate or DHB alone had no effect on the pattern of immunostaining. Photos are representative of sections from three different animals per condition. Scale bar = 10 μ m.

Staining for the NF- κ B subunit c-Rel (not shown) produced essentially identical results to the staining for p50 as described above. There were widespread increases after kanamycin treatment and a lack of localization in outer hair cell nuclei. Cotreatment with DHB or salicylate again promoted the nuclear translocation of c-Rel immunoreactivity into outer hair cell nuclei. Immunostaining with p65 increased in all cell types in the organ of Corti after kanamycin treatment, but no nuclear translocation was seen under any experimental conditions (not shown).

Kanamycin Treatment Changes Protein Levels, Phosphorylation, and Localization of I κ B α

On Western blots, total I κ B α , the inhibitory binding protein of NF- κ B, increased in the cochlea with kanamycin treatment, and this increase was prevented by cotreatment with salicylate (Fig. 7A). I κ B β showed a similar expression pattern in the cochlear homogenates (not shown). To assess the origin of the increasing levels of I κ B α after kanamycin treatment, mRNA levels of I κ B α were assayed in the cochlear homogenate. Results indi-

cated no difference of the mRNA levels of I κ B α between experimental groups (Fig. 7B). Phosphorylated tyrosine of I κ B α decreased with kanamycin treatment, however, and this decrease was reversed by concurrent injections of salicylate (Fig. 7C).

DISCUSSION

The salient result of this study is the finding that the NF- κ B pathway protects cochlear sensory cells from kanamycin-induced damage. In analyzing the NF- κ B cascade as a potential protective pathway against oxidant stress in the inner ear, we need to consider the differential toxic effects of the drug on the cells of the organ of Corti. In response to kanamycin treatment, the NF- κ B pathway is activated (i.e., NF- κ B is translocated into the nucleus) in supporting cells and inner hair cells, those cell types that will survive the kanamycin regimen. In contrast, outer hair cells, which are the primary targets of the toxic actions of kanamycin, lack this response and their nuclei remain devoid of NF- κ B. The notion of NF- κ B being associated with protective mechanisms is supported strongly by the

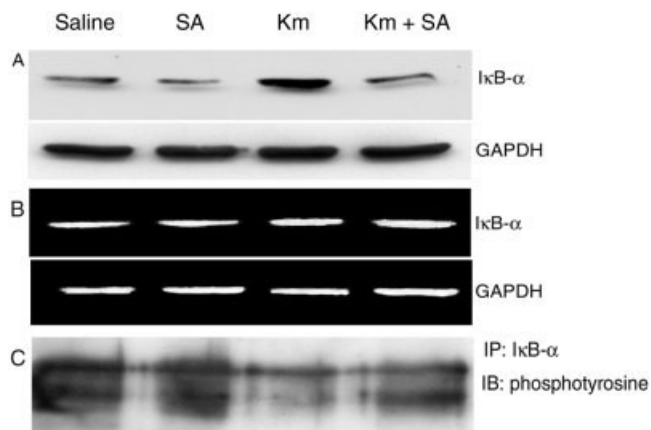


Fig. 7. Changes in IκBα. Cochlear homogenates were prepared for Western blotting and immunoprecipitation and total RNA was extracted for RT-PCR. **A:** Western blot. Salicylate alone (SA) induced no significant changes as compared to saline-injected animals (Saline). The levels of IκBα protein increased after kanamycin treatment (Km), whereas cotreatment with salicylate (Km + SA) returned the levels to those of controls. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is the standard for the amount of sample loaded onto the gel. The blot shown is representative of three independent assays; one additional animal showed no change of IκBα after kanamycin treatment. **B:** RT-PCR showed no difference in IκBα mRNA levels between treatment groups; GAPDH is a loading standard. The blot shown is representative of three independent assays. **C:** Cochlear extracts were immunoprecipitated with anti-IκBα antibody and immunoblotted for phosphotyrosine after separation by SDS-PAGE. The level of phosphotyrosine of IκBα was reduced after kanamycin treatment and restored by cotreatment with salicylate. One representative experiment of three is shown.

actions of DHB and salicylate, which prevent the structural and functional damage to outer hair cells (Song et al., 1997; Sha and Schacht, 1999a; Wu et al., 2002). These protective agents promote the translocation of NF-κB into the nuclei of outer hair cells. The activation of NF-κB as a survival pathway in aminoglycoside-induced hearing loss is in good agreement with a postulated survival function for NF-κB in a number of tissues under oxidative stress (Janssen-Heininger et al., 2000; Haddad, 2002; Lee and Choi, 2003).

The fact that kanamycin indeed produces oxidative stress in the cochlea is seen in the increase in 4-HNE labeling, indicative of lipid peroxidation. This cellular response is the *in vivo* correlate to *in vitro* studies demonstrating ROS formation by aminoglycosides in concert with transition metals and electron donors (Priuska and Schacht, 1995; Sha and Schacht, 1999a). In the context of aminoglycoside-induced oxidative stress, it is interesting that nitrotyrosine, a marker for peroxynitrite, was not elevated. Peroxynitrite had been implicated in the ototoxicity of gentamicin, but this claim was based solely on acute vestibular damage induced by local drug application (Takumida et al., 1999). The decreased tyrosine phosphorylation of IκBα also agrees well with the occurrence of oxidative stress in drug-induced ototoxicity, because it

is a redox-sensitive step, which can be triggered by prooxidative shifts in the intracellular environment (Hehner et al., 2000).

Because oxidative stress is evident in essentially all cochlear cells, the question arises why outer hair cells, but not other cell types, will succumb to kanamycin injury. This selective effect may be because outer hair cells are more susceptible to oxidant stress in general due to an intrinsically lower antioxidant capacity (Sha et al., 2001a). A resulting higher oxidant state of outer hair cells in response to kanamycin would favor oxidation of the Cys^{61/62} at the NF-κB subunits, resulting in an oxidized NF-κB complex with a low DNA-binding capacity (Toledano and Leonard, 1991). The continued presence (and increase) of NF-κB subunits in the cytoplasm of outer hair cells after kanamycin treatment indicates that translocation or DNA binding and subsequent gene expression is indeed inhibited. An elevated level of IκBα is a consequence of the decreased phosphorylation (and hence, decreased degradation) of IκBα and leads to retention of NF-κB in the cytoplasm. Salicylate reverses this effect of kanamycin and allows translocation of NF-κB into the nuclei of outer hair cells. In this context, it is interesting to consider that salicylate has been reported to inhibit NF-κB activation in cell culture (Yin et al., 1998; Yuan et al., 2001). This inhibition, however, was mediated by IκB kinase β, whereas IκB kinase α activity, which should be involved in the pathways investigated here, remained unaffected by aspirin and salicylate (Yin et al., 1998).

The most parsimonious explanation for the protective effect of DHB and salicylate is an action as radical scavengers reducing the ROS level in the cells. Given alone, these agents given do not activate any of the pathways that they restore in the combined injections with kanamycin; rather, they seem to remove the stimulus that causes the kanamycin-dependent changes, consistent with the reduction of lipid peroxidation in the cochlea. An antioxidant role of DHB or salicylate would be based on their capacity as iron chelators (Cohen et al., 2001) and hydroxyl radical scavengers (Coudray and Favier, 2000). Furthermore, the mediation of their protective effects via an antioxidant action is also consistent with the general efficacy of radical scavengers and iron chelators against aminoglycoside-induced otopathology, including deferrioxamine, lipoic acid, and mannitol (Song and Schacht, 1996; Song et al., 1997; Conlon et al., 1999). DHB or salicylate (and kanamycin as well) may therefore act as comodulators of NF-κB activation, a mechanism that has been proposed for ROS-induced responses in a number of cell types (Dröge, 2002). Such a comodulation can consist of subtle shifts in the oxidant state of cochlear cells after kanamycin treatment, which increases ROS formation (Sha and Schacht, 1999a), and cotreatment with DHB or salicylate, which attenuates this action of kanamycin.

In summary, signaling through NF-κB is an apparent survival pathway in the mammalian cochlea, modulated by the oxidant status of the cells. Because the adverse actions of other ototraumatic agents, such as noise or the antineo-

plastic agent cisplatin, have also been linked to ROS formation, this pathway may be of general importance in maintaining the integrity of the inner ear. This insight may not only help our understanding of cochlear pathology but also provide for new approaches to the preservation of sensory cells.

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