Pivotal role of Harakiri in the induction and prevention of gentamicin-induced hearing loss

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Gentamicin is a widely used ototoxic agent. In this study, we shed light on the mechanisms underlying gentamicin-induced hearing loss. More importantly, we demonstrate in vivo and in vitro the effectiveness of a strategy for preventing drug-induced hearing loss using L-carnitine (LCAR), a safe micronutrient that plays a key role in energy metabolism and detoxification [Rebouche, C. J. & Seim, H. (1998) Annu. Rev. Nutr. 18, 39–61]. We show that LCAR prevents changes in hearing threshold and cochlear damage in newborn guinea pigs exposed to gentamicin in utero. Mechanically, gentamicin-induced apoptosis of auditory cells is mediated by the extracellular signal-regulated kinase (ERK) 1/2 mitogen-activated protein kinase (MAPK) pathway through up-regulation of the proapoptotic factor Harakiri (Hrk). Most important, small interfering RNA (siRNA) experiments demonstrate that Hrk up-regulation is crucial for gentamicin-induced apoptosis. LCAR, in contrast, prevents both gentamicin-induced Hrk up-regulation and apoptosis acting by means of c-Jun N-terminal kinase (JNK). Together, these results outline pathways for gentamicin-induced hearing loss and its prevention and assign a key role to Hrk in these processes. Thus, our data offer a conceptual framework for designing clinical trials using a safe micronutrient, LCAR, as a simple preventive strategy for iatrogenically induced ototoxicity.

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Abbreviations: LCAR, L-carnitine; Hrk, Harakiri; ABR, auditory brainstem response; SEM, scanning electron microscopy; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; siRNA, small interfering RNA; GC, outer hair cell; MAPK, mitogen-activated protein kinase; p, phosphorylated; l, inhibitor.

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(gentamicin plus LCAR). A total of 71 puppies were born, and newborn mortality was defined as either stillborn or death within the first 48 h of life. Two weeks after delivery, mothers and newborns were tested by auditory brainstem response (ABR) and immediately euthanized with CO2. Cochleae were then harvested for light microscopy (frozen sections), confocal microscopy, and scanning electron microscopy (SEM) studies. In a particular series of experiments, 3-week-old guinea pigs \((n/11005)4\) received a single i.p. injection of either saline solution or gentamicin \((400 \text{ mg/kg of body weight})\) and were euthanized 6 h or 24 h later. Cochleae were then harvested and processed for light microscopy.

**ABR.** All experiments were performed inside an acoustically insulated booth (Industrial Acoustics, Bronx, NY). Guinea pigs were anesthetized by i.p. injections of 60 mg/kg ketamine and 5 mg/kg xylazine. The subject’s core body temperature was maintained with a thermostatically controlled heating blanket (Baxter K-MOD 100). Broadband clicks (10-ms duration, 50-μs sample rate) were generated by a computer-controlled BioSig Amplifier (SIGGEN Computer Software, Tucker Davis Technologies, Alachua, FL), calibrated with a digital sound level meter (RS 33-2055), and presented alternately to the left and right auditory meatus through an ear bar connected to a Beyer DT-48 transducer. Platinum subdermal needle electrodes (F-E2, Grass Instruments, West Warwick, RI), fed by an Opti-Amp 3000 bioamplifier (Intelligent Hearing Systems, Miami) with a gain of 100,000 and a 300-Hz to 5-kHz filter setting, were placed under each auricle and at the vertex. Averaged responses from 512 stimuli were obtained at 5-dB intervals in the range from 80 dB to 0 dB. Auditory thresholds, defined as the lowest intensity to yield a reproducible deflection in the evoked response trace, were estimated for each ear, and threshold shifts were evaluated by comparison of the means calculated for each experimental group with respect to the control by using ANOVA techniques with Bonferroni correction factor for multiple comparisons (STATVIEW 4.1 and SUPERANOVA, Abacus Concepts, Berkeley, CA).

**Frozen Sections.** Otic bullae were fixed with 4% paraformaldehyde overnight at 4°C, washed out with PBS for 30 min, and decalcified with 120 mM EDTA for 4 weeks. Samples were washed with PBS twice for 30 min and then placed in 15% sucrose solution for 20 min and in 30% sucrose solution at 4°C overnight. Next, samples were molded with OCT compound embedding medium (Sakura Finetek, Torrance, CA) in proper orientation, sectioned in a cryostat at 10-μm thickness, and stored at −20°C. Sections were labeled with anti-Hrk (Q-17, Santa Cruz Biotechnology), anti-phosphorylated \((p)-\text{ERK1/2}\), and anti-\(p\)-JNK (Cell Signaling Technology, Beverly, MA) at 1:100 dilution following standard protocols, and observed with a Zeiss Axiovert 135 TV inverted microscope with Neo-fluar \(\times 10, \times 20, \times 40\) objectives.

**Confocal Microscopy and SEM.** For confocal microscopy, otic bullae were opened and fixed in 3% paraformaldehyde (PF) in PBS (pH 7.4) for 1 h. Next, cochlear turns were separated with small scissors and individually processed. Samples were permeabilized...
with 0.5% Triton X-100 in PBS for 30 min, followed by another 30-min incubation in blocking solution (10% goat serum plus 1% BSA in PBS). Finally, samples were incubated at 37°C for 30 min with 33 nM rhodamine/phalloidin (Molecular Probes) diluted in PBS from a stock solution of 3.3 µM in methanol (100 units/ml), mounted, and observed. HEI-OC1 cells, in turn, were fixed in 4% PF for 1 h at 4°C and then washed with PBS three times for 5 min each. Primary antibodies were used at 1:100 dilution in PBS plus 1% Tween 20 (PBST) in overnight incubations at 4°C. Gentamicin uptake was monitored with a monoclonal antibody against gentamicin (Fitzgerald Industries International, Chelmsford, MA). Anti-mouse and anti-rabbit FITC-, CY2-, and CY3-bound secondary antibodies (Jackson ImmunoResearch) were used at 1:1,000 dilutions in PBST in 1-h incubations at room temperature. Annexin V/propidium iodide labeling was performed by using the Vybrant Apoptosis Assay Kit No. 2 (Molecular Probes) following the manufacturer's protocol. Samples were observed with a Zeiss LSM-410 laser confocal microscope with objectives C-Apo ×40 and ×63 (N.A. = 1.2). For SEM, otic bullae were opened and fixed by immersion in 2.5% glutaraldehyde for 4 h. Next, they were decalcified for 3–5 days in 120 mM EDTA (Sigma), washed with PBS, dissected out to expose the organ of Corti, reimmersed in 2.5% glutaraldehyde, and sequentially exposed to tannic acid, osmium tetroxide, and thiocarbohydrazide as described (11). Finally, samples were dried by using a critical-point dryer, and examined in an FE-SEM (XL30 S-FEG, FEI-Philips, Hillboro, OR).

Evaluation of Drug-Induced Cochlear Damage. SEM and confocal samples of guinea pig cochlea were examined thoroughly, and the percentage of missing hair cells was obtained by dividing the number present by the total number counted plus the scars showing missing hair cells × 100. Results were evaluated with ANOVA techniques by using arcsin transformation of the data.

Caspase-3 Activation. HEI-OC1 cells were cultured at 33°C, 10% CO2 in DMEM (GIBCO/BRL) supplemented with 10% FBS (GIBCO/BRL) without antibiotics, in uncoated dishes 100 mm in diameter (12). Untreated cells and cells exposed to 50 µM gentamicin (Sigma) for 24 h, with and without a 48-h preincubation with 2 µg/ml LCAR (Sigma), and with and without PD98059 [extracellular signal-regulated kinase (ERK) inhibitor (ERK-I) 100 µM] and SP600125 [c-Jun N-terminal kinase (JNK) inhibitor (JNK-I) 5 µM] (both from Calbiochem), were used in caspase-3 activation assays (CaspACE Assay System, Promega), following the manufacturer’s protocols. Absorbance at 405 nm was measured in 96-well plates (flat-bottom) by using the computer-controlled microplate reader GENios (Tecan, Research Triangle Park, NC) with MAGELLAN 5.0 software.

Western Blotting. Cells were lysed at 4°C in a 50 mM Tris buffer solution (pH 7.4) containing 1% Nonidet P-40, 2 mM EDTA, 100 mM NaCl, 1 mM vanadate, 10 µl/ml 0.1 M PMSF, 2 µl/ml 10 mg/ml leupeptin, and 2 µl/ml 10 mg/ml aprotinin. Samples were mixed with loading buffer (2 g of SDS/0.002 g of bromophenol blue/1.54 g of DTT/8 ml of 1 M Tris (pH 6.8)/10 ml of glycerol), heated at 95°C for 5 min, analyzed by SDS/PAGE gels (30 µg of protein per lane), transferred to poly(vinylidene difluoride) (PVDF) membranes, and incubated with primary antibodies. The reaction was detected by ECL (Amersham Pharmacia) by using peroxidase-labeled secondary antibodies.

Gene Profiling. HEI-OC1 cells growing to confluence in 100-µm plastic culture dishes (six per experimental condition) were incubated with 50 µM gentamicin for 24 h at 33°C. Total RNA was extracted from cells by homogenization in TRIzol LS Reagent (Gibco-Invitrogen) following the manufacturer’s protocol. Biotin-dUTP-labeled cDNA probes were generated by PCR, added to prehybridized GEArray membranes (GEArray Q series Mouse Apoptosis and Stress & Toxicity PathwayFinders, SuperArray, Frederick, MD), and incubated in a hybridization oven overnight. Next, membranes were incubated with the streptavidin-AP conjugate and developed with the CDP-Star chemiluminescent substrate provided by the manufacturer following GEArray protocols. Changes in gene expression were validated by Western blotting and RT-PCR. For RT-PCR analysis, RNA was extracted from HEI-OC1 cells with TRIzol, and cDNA was prepared from the RNA by using SuperScript (Invitrogen). The following oligonucleotide pairs were used to amplify Hrk-specific transcripts from the normal cell line cDNA: for Hrk, (+) 5'-ATT CGG TAC CTG TGC ATG CCT GCT G-3' and (-) 5'-TGT GCT GAA CAG TTG TGC CAC G-3'; for GAPDH (control for RNA integrity), (+) 5'-TGA TGA CAT CAA GAA GTG GTG AAG-3' and (-) 5'-TCC TG TGG GAG GCC ATG TAG GCC AT-3'. PCR was carried by using the following conditions: 20 cycles of denaturation at 95°C for 30 s, 40 cycles of annealing at 57°C for 30 s, and 40 cycles of extension at 72°C for 30 s.
annealing 52°C for 30 s, and extension at 72°C for 1 min. PCR product was analyzed by separation on a 2% agarose-TAE gel.

Small Interfering RNA (siRNA). HEI-OC1 cells were transfected with four SMARTselection-designed siRNA oligonucleotides targeting Hrk, alone and pooled (Dharmacon Research, Lafayette, CO). The oligonucleotide sequences are as follows: 1, GTAAAGAGCTGATGGTGGA; 2, GATGTGAACTCTGAGACTT; 3, AAACTTACATGGACCGGTG; and 4, GAACTCTGAGACTTCGTAA. For ectopic reconstitution of the Hrk expression, we used a Hrk-resistant (rHrk) cDNA, kindly provided by G. Nunez (University of Michigan, Ann Arbor), that lacks the siRNA-targeting sequences (12). HEI-OC1 cells were grown in six-well plates at 33°C until 90–95% confluent, and then incubated with Lipofectamine 2000 (Invitrogen), with and without the siRNA's oligos, for 4 h following the manufacturer’s protocol. Next, the transfection mixture was replaced with complete growth medium, and the cells were cultured for another 48 h. Finally, cells were exposed to gentamicin for 24 h and then collected and processed for Western blotting and caspase-3 activation assays as described above.

Results and Discussion

We used guinea pigs at late stages of pregnancy as an animal model to investigate gentamicin-induced hearing loss and the effect of LCAR supplementation on the newborns. Interestingly, we observed a significant difference in neonatal mortality rates among the groups included in our study (Fig. 1a). Although exposure to gentamicin increased mortality of newborn guinea pigs, LCAR supplementation, both before and simultaneously with gentamicin, significantly decreased it. In addition, although it may be presumed that the animals potentially most affected by gentamicin were those stillborn, ABR experiments showed a significant gentamicin-induced increase in the hearing threshold of the survivors (gentamicin/H11005 30 dB vs. control/H11005 21 dB, P ≤ 0.01, Fig. 1b). LCAR supplementation, either from 28 days of pregnancy or coincidental with gentamicin injections, completely prevented this change (LCAR plus gentamicin/H11005 23 dB and gentamicin plus LCAR/H11005 21 dB). A similar protective effect of LCAR was observed in the mothers (Fig. 5, which is published as supporting information on the PNAS web site). Importantly, this response would be indeed an otoprotective effect, because LCAR does not interfere with the antibiotic efficacy of gentamicin (Fig. 6, which is published as supporting information on the PNAS web site).

SEM and confocal techniques confirmed both that gentamicin induces a significant damage of outer hair cells (OHCs) and that this damage can be prevented by LCAR supplementation in newborn guinea pigs (Fig. 1d–i). Drug-induced OHC death was similar in the first three turns of the cochlea (Fig. 1d and g–i). The apical fourth turn (low frequency region), however, was remarkably affected by gentamicin, with near disappearance of the normal pattern of three parallel rows of OHCs and many OHCs showing single, giant stereocilia or disorganized hair...
and potassium-induced apoptosis of cerebellar granule neurons recently implicated in axotomy-induced neuronal cell death (16).

Hrk expression in HEI-OC1 cells was already augmented after a 6-h incubation with gentamicin, and there were further increases with longer incubation times (Fig. 3a). In contrast, preincubation of the cells with LCAR abolished this response. Most importantly, suppression of Hrk expression by siRNA abolished the gentamicin-induced activation of caspase-3, a reliable indicator of apoptosis, in cultured auditory cells. Moreover, gentamicin also increased the expression of Hrk in OHCs and other cell populations of the guinea pig cochlea, and this increase was prevented by LCAR supplementation (Fig. 7a–c), which is published as supporting information on the PNAS web site. Therefore, LCAR protects auditory cells from apoptosis by preventing the gentamicin-induced up-regulation of Hrk.

Subsequent studies on signaling cascades that can mediate the gentamicin-induced transcriptional regulation of Hrk revealed a role for mitogen-activated protein kinases (MAPKs). Pharmacological inhibition of ERK1/2 prevented gentamicin-induced activation of caspase-3 in HEI-OC1 cells (control = 100 ± 4% vs. gentamicin plus ERK-I = 160 ± 20%, P = not significant), whereas inhibition of JNK increased the apoptotic effect of gentamicin (gentamicin = 280 ± 20% vs. gentamicin plus JNK-I = 510 ± 30%, P = 0.001) (Fig. 4a). Moreover, inhibition of ERK1/2, but not JNK, significantly reduced gentamicin-induced Hrk expression in auditory cells (Fig. 4b). These results suggest that gentamicin-induced apoptosis is mediated by ERK1/2. Consistently, studies of MAPK activation indicated...
that gentamicin induces both phosphorylation and nuclear translocation of ERK1/2 (Fig. 4 c–f), a process associated with neuronal apoptosis and neurodegeneration (18). JNK phosphorylation, on the other hand, diminished with 6 h incubation, and it is abolished by 24 h incubation with gentamicin (Fig. 4c). Remarkably, a similar gentamicin-induced ERK activation occurs in OHCs and other cochlear cell populations of guinea pigs exposed to gentamicin (Fig. 8 a–c), which is published as supporting information on the PNAS web site. JNK, in contrast, is weak and transiently activated mainly in supporting cells (Fig. 8 d–f).

Interestingly, inhibition of JNK, but not ERK, abolished the protective effects of LCAR (gentamicin = 280 ± 20% vs. LCAR plus gentamicin = 88 ± 6%, P ≤ 0.03; vs. LCAR plus gentamicin plus JKNI = 244 ± 6%, P = not significant; vs. LCAR plus gentamicin plus ERK-I = 112 ± 8, P ≤ 0.05; Fig. 4d). Consistently, inhibition of JNK completely reversed the preventive effect of LCAR on gentamicin-induced up-regulation of Hrk (Fig. 4a and b), and LCAR ameliorated the gentamicin-induced inactivation of JNK (Fig. 4c). These results suggest that, whereas gentamicin-induced up-regulation of Hrk in auditory cells is mediated by ERK1/2, the preventive effects of LCAR occur via JNK.

Altogether, these results demonstrate that different MAPKs play antagonistic roles in gentamicin cell toxicity, setting an important baseline for further defining the effect of LCAR on these important pathways. They are also consistent with reports in the literature identifying MAPKs as important mediators in the apoptotic pathways activated by ototoxic drugs such as neomycin and cisplatin (19–22). However, it is essential to note that, although neomycin, like gentamicin, is an aminoglycoside antibiotic, these drugs induce different cellular and biochemical responses. Noteworthy, for instance, whereas neomycin is mainly cochleotoxic, gentamicin is considered more a vestibulotoxic agent. Ylikoski et al. (23) have previously suggested that JNKs, not ERK, could be mediating in the gentamicin-induced death of inner hair cells of the cochlea and type I hair cells of the vestibular organ in guinea pigs. However, as recognized by the authors, the cochlear damage induced by gentamicin in their study was so extensive as to prevent the actual documentation of JNK activation. Similarly, the proportion of hair cell death associated with necrosis (versus apoptosis) was unknown. Therefore, the conditions reported in the current study have allowed a more defined determination of the role of JNK in gentamicin-induced apoptosis and provide a reliable reference for future studies aimed at evaluating the efficacy of novel compounds that manipulate this pathway.

The findings that Hrk is expressed in auditory cells and the functional characterization of this molecule as a mediator of gentamicin-induced apoptosis, as reported here, are significantly important for better understanding the molecular repertoire that is involved in regulating cell death in the inner ear. The fine details of the mechanisms underlying the proapoptotic effects of Hrk are poorly understood. The dominant theory is that Hrk would inhibit the antiapoptotic function of other Bel-2 family member by heterodimerizing with them. However, a homotetrameric protein, p32, was recently isolated in a two-hybrid screen by its ability to interact with Hrk (24). Hrk-mediated apoptosis requires tetrameric p32 to form a channel in the mitochondria membrane and destabilize the function of this organelle. Thus, the emerging picture describing the functional mechanisms of Hrk suggests that several protein–protein interactions are necessary for its effects. Thus, it is likely that these types of interactions also underly the effects of Hrk in gentamicin-induced apoptosis. Future studies focused on characterizing these signaling pathways downstream of Hrk in auditory cells could provide additional therapeutic targets to prevent iatrogenic hearing loss.

In summary, we have used a combination of both animal and cellular models to further investigate the molecular mechanisms underlying gentamicin-induced ototoxicity and to define an effective chemopreventive strategy for this phenomenon. We presented evidence that supplementation of pregnant mothers with LCAR prevents neonatal mortality and sensorineural hearing loss induced by gentamicin in newborn guinea pigs. Our experiments with auditory cells outline a more detailed pathway for gentamicin cell toxicity mediated by activation of the ERK1/2 and inhibition of the JNK pathways, followed by the translocation of ERK to the nucleus, transcriptional up-regulation of Hrk, and initiation of the execution phases of apoptosis. LCAR, on the other hand, would be preventing the gentamicin-induced inhibition of JNK and the consequent up-regulation of Hrk, blocking cell death. Thus, L-carnitine, a natural neuroprotective agent that can be safely used in humans (25), could be central for developing clinical strategies to prevent gentamicin-induced hearing loss.

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