Hsp70 inhibits aminoglycoside-induced hearing loss and cochlear hair cell death

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Abstract Sensory hair cells of the inner ear are sensitive to death from aging, noise trauma, and ototoxic drugs. Ototoxic drugs include the aminoglycoside antibiotics and the antineoplastic agent cisplatin. Exposure to aminoglycosides results in hair cell death that is mediated by specific apoptotic proteins, including c-Jun N-terminal kinase (JNK) and caspases. Induction of heat shock proteins (Hsps) can inhibit JNK- and caspase-dependent apoptosis in a variety of systems. We have previously shown that heat shock results in robust upregulation of Hsps in the hair cells of the adult mouse utricle in vitro. In addition, heat shock results in significant inhibition of both cisplatin- and aminoglycoside-induced hair cell death. In this system, Hsp70 is the most strongly induced Hsp, which is upregulated over 250-fold at the level of mRNA 2 h after heat shock. Hsp70 overexpression inhibits aminoglycoside-induced hair cell death in vitro. In this study, we utilized Hsp70-overexpressing mice to determine whether Hsp70 is protective in vivo. Both Hsp70-overexpressing mice and their wild-type littermates were treated with systemic kanamycin

(700 mg/kg body weight) twice daily for 14 days. While kanamycin treatment resulted in significant hearing loss and hair cell death in wild-type mice, Hsp70-overexpressing mice were significantly protected against aminoglycoside-induced hearing loss and hair cell death. These data indicate that Hsp70 is protective against aminoglycoside-induced ototoxicity in vivo.

Keywords Cochlea · Hearing loss · Ototoxicity · Aminoglycoside · Kanamycin · Hair cell

Introduction

Aminoglycosides are a class of antibiotics used in the treatment of serious gram-negative bacterial infections. Due to their low cost and high efficacy, aminoglycosides are among the most commonly prescribed antibiotics worldwide, despite their serious side effects of ototoxicity and nephrotoxicity (Forge and Schacht 2000). Aminoglycosides include streptomycin, gentamicin, neomycin, kanamycin, tobramycin, and amikacin. Approximately 15–20% of patients receiving aminoglycosides experience significant hearing loss and/or balance disturbances (Forge and Schacht 2000). This toxicity is due to aminoglycoside-induced death of the mechanosensory hair cells of the auditory and vestibular systems in the inner ear. Since mammalian hair cells are not regenerated, aminoglycoside-induced hearing loss and/or vestibular deficits are permanent. Safe administration of aminoglycosides necessitates therapeutic interventions aimed at preventing the unwanted side effects of these otherwise efficacious antibiotics.

Studies in rats have shown that aminoglycosides can be found in the inner ear only minutes after systemic administration and levels plateau within 30 min to 3 h

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(Tran Ba Huy et al. 1986). Clearance of aminoglycosides from both inner ear fluids and tissues is very slow: While the half-life of aminoglycosides in serum is about 3–5 h, they can remain in the inner ear for longer than 30 days (Tran Ba Huy et al. 1986). When exposed to aminoglycosides, hair cells demonstrate both morphologic and molecular features of apoptotic cell death, including condensed, marginated chromatin, and positive terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling staining (Forge and Fradis 1985; Li et al. 1995; Lang and Liu 1997; Nakagawa et al. 1998; Forge and Li 2000; Matsui et al. 2002; Mangiardi et al. 2004). In addition, aminoglycoside-induced hair cell apoptosis is mediated by c-Jun N-terminal kinase (JNK), a member of the mitogen-activated protein kinase family. Inhibition of JNK using a variety of methods inhibits aminoglycoside-induced hair cell death both in vitro and in vivo (Pirvola et al. 2000; Ylikoski et al. 2002; Matsui et al. 2004; Sugahara et al. 2006; Eshraghi et al. 2007). Furthermore, aminoglycoside exposure results in cytochrome c release from the mitochondria and caspase activation in hair cells (Cheng et al. 2002; Cunningham et al. 2002; Matsui et al. 2004; Sugahara et al. 2006). Broad spectrum inhibition of caspases inhibits aminoglycoside-induced hair cell death, both in vitro and in vivo (Cunningham et al. 2002; Matsui et al. 2002; Cheng et al. 2003; Matsui et al. 2003, 2004), suggesting that this cell death is mediated by caspases.

Induction of heat shock proteins (Hsps) in response to stress—chemical, physical, or toxic—is a highly conserved response that is cytoprotective in a wide variety of systems (Martindale and Holbrook 2002). Hsps function as molecular chaperones by promoting proper folding of nascent and/or denatured polypeptides, preventing protein aggregation, and trafficking proteins to their appropriate subcellular localizations (Morimoto 2008). Stress results in transcriptional upregulation of Hsps via activation of the heat shock transcription factor 1 (Hsf1; Ankar and Sistonen 2007). In addition to their functions as molecular chaperones, Hsps have also been shown to inhibit apoptosis. Hsp70 is the most highly conserved and most stress-inducible Hsp. In other systems, Hsp70 has been shown to inhibit some of the same pathways that mediate aminoglycoside-induced hair cell death, including JNK activation and/or JNK-mediated cell death, cytochrome c release, and caspase activation (Mosser et al. 1997; Jaattela et al. 1998; Beere et al. 2000; Tsuchiya et al. 2003).

In a previous study, we utilized an organ culture preparation of the adult mouse utricle, a vestibular organ of the inner ear to examine the role of Hsp70 in heat shock-induced protection against aminoglycoside-induced hair cell death in vitro. The utricle model system is the best-characterized in vitro preparation of mature mammalian sensory hair cells (Matsui and Cotanche 2004;

Chiu et al. 2008). Heat shock leads to robust upregulation of Hsp70 in hair cells and inhibits aminoglycoside-induced hair cell death (Cunningham and Brandon 2006; Taleb et al. 2008). This protective effect of heat shock against aminoglycoside-induced hair cell death is not observed in utricles from *Hsp70*^{−/−} mice, indicating that Hsp70 is necessary for the protective effect of heat shock (Taleb et al. 2008). Furthermore, utricles from mice that constitutively overexpress Hsp70 are protected from aminoglycoside-induced death in vitro (Taleb et al. 2008). These data indicate that Hsp70 inhibits aminoglycoside-induced hair cell death and is necessary for the protective effect of heat shock. The goal of the current study was to determine whether Hsp70 overexpression is protective against aminoglycoside-induced hearing loss and hair cell death in vivo.

Methods

Animals

Mice were maintained in the central animal care facility at the Medical University of South Carolina (Charleston, SC, USA). Hsp70-overexpressing mice constitutively express rat Hsp70 under the control of the human cytomegalovirus immediate early enhancer (CMV-IE) and chicken β -actin promoter (Marber et al. 1995). These transgenic mice are on a BALB/c $\times$ C57BL/6 background. Male Hsp70-overexpressing mice were mated with wild-type female CB6F1 mice obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Resulting litters were approximately 50% wild-type (transgene negative; homozygous) and 50% Hsp70-overexpressing mice (transgene positive; heterozygous). All animal protocols were approved by the MUSC Institutional Animal Care and Use Committee. All mice were euthanized with an overdose of Nembutal (Abbott Laboratories, USA).

Experimental groups and design

Twenty-two mice (10 Hsp70-overexpressing mice and 12 wild-type littermates) were used for this study. Mice were 4 weeks old at the beginning of the study, and animals of both genders were used. Each animal was randomly assigned to an experimental group (saline-injected or kanamycin-injected). Mice underwent hearing testing via auditory brainstem response (ABR) threshold determination 1 day prior to the onset of the kanamycin (or saline) treatment (pretest), and ABR testing was repeated 3 weeks after the completion of the drug regimen (posttest). Immediately after the posttest ABR, mice were euthanized, and tissue was collected for immunochemistry.

Genotyping

Twenty-one days after birth, pups were weaned and genotyped. Tail clips (1 mm) were lysed using DirectPCR Lysis Reagent (Viagen Biotech #101-T, Los Angeles, CA, USA). Crude DNA obtained from tail lysates was used in one-step polymerase chain reaction genotyping (TaqMan Core Reagent Kit, Applied Biosystems #N808-0228, Foster City, CA, USA; Mastercycler, Eppendorf #5333, Hamburg, Germany). The following primers were used: forward primer 5'-ATTACGGGGTCATTAGTTCATAGCC-3', reverse primer 5'-GTAGGAAAGTCCCATAAGGTCATGT-3', yielding a 280-bp product of the CMV-IE region of the rat *Hsp70* transgene.

Auditory brainstem response threshold determination

Prior to kanamycin (or saline) injections, mice were pretested for normal evoked potential thresholds. These thresholds were used as baseline measurements for deriving the threshold shifts caused by kanamycin. Mice were anesthetized with an intraperitoneal injection of 90 mg/kg body weight of ketamine hydrochloride (Fort Dodge Animal Health, Dodge, IO, USA) and 2.3 mg/kg body weight of xylazine hydrochloride (Ben Venue Laboratories, Bedford, OH, USA). During ABR measurements, mice were kept on a warming pad to maintain body temperature (Deltaphase Isothermal Pad, Braintree Scientific #39DP, Braintree, MA, USA). Equipment for ABR measurements was obtained from Tucker-Davis Technologies (TDT System 3; Alachua, FL, USA). ABRs were recorded using platinum subcutaneous needle electrodes (Grass Technologies #FH-E2, West Warwick, RI, USA). The noninverting electrode was inserted on the midline at the vertex. The inverting electrode was inserted over the mastoid of the right ear. The common electrode was placed subcutaneously in the left upper leg. The signals were generated using TDT System 3 modules (sampling rate 50 kHz). Tone bursts (3 ms duration, 1 ms rise/fall times) were presented using a free-field loudspeaker (Tucker-Davies Technologies #FF1) at 4, 8, 11.3, 16, 22.4, and 32 kHz. The loudspeaker was at 0° azimuth, and the distance between the mouse (nose) and the loudspeaker was 2.5 cm. Stimuli were presented at a rate of 31/s. Responses were obtained using a RA4PA 4-channel Medusa preamplifier (TDT). Filter settings were 300 Hz (high pass), 3 kHz (low pass), and at 60 Hz (notch). ABRs were first recorded at 95–105 dB sound pressure level (SPL), and stimulus intensity was reduced in 10 dB steps until the response was no longer identifiable. Stimulus intensity was then increased in 5 dB steps until an identifiable and repeatable response with a latency of less than 12 ms was present. Threshold responses were compared to suprathreshold waveforms, and all traces

were evaluated by a certified audiologist. For threshold determination, responses to 1,000 stimulus presentations were recorded for each of at least two trials. Following ABR pretesting, animals remained on the warming pad until they had recovered from anesthesia. For posttest ABRs, animals with no detectable response at the output limits of the equipment were assigned a “threshold” value of 5 dB above the output limits of the loudspeaker (i.e., 105 dB if the output limit was 100 dB). Following posttest ABR recordings, animals were euthanized without recovering from anesthesia.

Kanamycin treatment

Kanamycin sulfate was obtained from USB Corporation (Cleveland, OH, USA). According to the manufacturer, kanamycin activity accounted for 78.2% of this product's weight (i.e., corrected for sulfate and water). Kanamycin sulfate powder was therefore dissolved in sterile saline at a final concentration of 45 mg/ml so that a dose of 700 mg/kg kanamycin base could be delivered to each mouse by injecting a volume of 0.02 ml/g body weight. Kanamycin (or saline) treatment was initiated 1 day after initial ABR assessment. Hsp70-overexpressing mice ($n=5$) and wild-type littermates ($n=6$) received kanamycin (700 mg/kg body weight) subcutaneously twice daily for 14 days. Control Hsp70-overexpressing mice ($n=5$) and control wild-type littermates ($n=6$) received subcutaneous saline injections twice daily for 14 days. The first injection of each day was administered between 7 A.M. and 8 A.M., and the second injection was administered 8 h later. This protocol was slightly modified from Wu et al. (2001). Mice of both genotypes tolerated the kanamycin injections well. All mice ate and drank normally. Each mouse was weighed daily, and all mice continued to gain weight throughout the treatment period. One kanamycin-injected animal died during the experiment, but necropsy data failed to show overt signs of kanamycin-induced nephrotoxicity. No animals displayed overt signs of vestibular disturbance such as head tilt, spinning, or rolling. Three weeks after the last injection, animals again underwent ABR threshold testing using the above protocol.

Immunohistochemistry

After each animal was euthanized and decapitated, the bony auditory bulla was opened, and the stapes was removed from the oval window. The bony apex of the cochlea was opened using a scalpel blade. Cochleae were perfused with ice-cold 4% paraformaldehyde through the small apical opening. Utricles were then removed via a small opening between the VIIIth nerve root and the semicircular canals. Utricles were then fixed with 4% paraformaldehyde overnight at 4°C.

Whole-mount cochlear half-turns

Cochleae were dissected from the auditory bullae and hair cells were labeled immunohistochemically using a protocol that was slightly modified from Heydt et al. (2004). Following perfusion with 4% paraformaldehyde (above), cochleae were immersion fixed in the same fixative for 2 h at room temperature while rocking. Cochleae were washed with phosphate buffered saline (PBS) and then incubated in 0.5 M ethylenediaminetetraacetic acid for 3 days. Cochleae were washed with PBS and bisected on the midmodiolar plane using a razor blade.

Additional staining steps were all carried out at 4°C on a nutator. Quenching of endogenous peroxidase was achieved by incubating cochleae in 3% hydrogen peroxide/37% water/50% PBS/10% methanol for 2.5 h. Cochleae were then incubated in blocking solution (2% bovine serum albumin/0.8% normal goat serum/0.4% Triton-X in PBS) overnight. Polyclonal antimyosin VIIa (Proteus BioSciences Inc. #25-6790, Ramona, CA, USA) was used as a hair cell marker and was diluted 1:30 in blocking solution. Cochleae were incubated in primary antibody solution for 4 days and then washed with blocking solution for 4 h. Biotinylated goat antirabbit secondary antibody (Vector Laboratories Inc. #PK-4001, Burlingame, CA, USA) was diluted 1:100 in blocking solution. Cochleae were incubated in secondary antibody solution overnight, washed with PBS, and then incubated in ABC (Vector Labs #PK-4001) overnight. Following another wash with PBS, cochleae were incubated in buffer (Vector Laboratories Inc. #SK-4100) for 20 min prior to incubation in diaminobenzidine (Vector Laboratories Inc. #SK-4100) for 6 h. Cochleae were washed with PBS before proceeding with the fine tissue dissection. Using a microscalpel and iridectomy scissors, hemicochleae were dissected into apical, middle, and basal half-turns. The osseous spiral lamina, modiolus, stria vascularis, and tectorial membrane were removed. Cochlear half-turns were mounted on slides using Fluoromount-G (Southern Biotech).

Whole-mount utricles

The adult mouse utricle preparation has previously been described in detail (Cunningham 2006). There are two types of hair cells in the utricle. Type I hair cells are flask-shaped and are contacted by large afferent nerve calyces (Desai et al. 2005). Type II hair cells are more cylindrical and are generally contacted by smaller bouton afferents (Desai et al. 2005). While both type I and type II hair cells are found throughout the utricle, type I hair cells are most prevalent in a central comma-shaped region called the striola. In order to visualize both types of hair cells, utricles were double-labeled using antibodies against two calcium-

binding proteins. Anticalmodulin labels all of the hair cells in the utricle, while anticalbindin labels only type I hair cells (Cunningham and Brandon 2006). This double-label protocol thus allows for differential counts of striolar vs. extrastriolar hair cells.

Adult mouse utricles were dissected under sterile conditions as previously described (Cunningham 2006). Utricles were fixed in 4% paraformaldehyde overnight at 4°C and then washed with PBS. Otoconia were removed via a stream of PBS using a syringe with a fine gauge needle. Utricles were incubated in blocking solution (2% bovine serum albumin/0.8% normal goat serum/0.4% Triton-X in PBS) for 3 h at room temperature. Primary antibodies included monoclonal anticalmodulin (Sigma #C 3545; 1:150) and polyclonal anticalbindin (Chemicon #AB1778, Temecula, CA, USA; 1:200), which were diluted in blocking solution and applied simultaneously at 4°C overnight. Secondary antibodies were Alexa 488 conjugated goat antimouse IgG (Invitrogen #A11001; 1:500) and Alexa 594 conjugated goat antirabbit IgG (Invitrogen #A11012; 1:500). These were diluted in blocking solution and applied for 4 h in the dark at room temperature on a rocking platform. Utricles were whole mounted on glass slides using Fluoromount-G (Southern Biotech, Birmingham, AL, USA).

Fluorescent microscopy and hair cell counts were performed using a Zeiss Axioplan 2 fluorescent microscope, a high-resolution monochrome digital camera (Zeiss Axiocam MR), and Zeiss AxioVision software. Eight 900 μm^2 (30×30 μm) areas were selected for hair cell counts in each utricle. Hair cells were counted in each square. The counted areas included four striolar (calmodulin- and calbindin-positive) and four extrastriolar (calmodulin-positive, calbindin-negative) regions per utricle. Hair cell counts from the four extrastriolar and four striolar regions were averaged separately. Hair cell counts are reported as the striolar and/or extrastriolar hair cell density for each utricle.

Statistical analyses

Data were analyzed by either one-way or two-way analysis of variance (ANOVA) using statistical software SYSTAT 8.0 (San Jose, CA, USA). Post hoc tests were performed using pooled variances. Differences are reported as significant if $p < 0.05$.

Results

Hsp70 overexpression inhibits aminoglycoside-induced hearing loss

Hsp70 overexpression inhibits aminoglycoside-induced vestibular hair cell death in organ culture (Taleb et al.

2008). To determine whether Hsp70-overexpressing mice are protected against aminoglycoside-induced hearing loss, both Hsp70-overexpressing mice and their wild-type littermates received either kanamycin (700 mg/kg of body weight) or saline injections twice daily for 14 days. Pretest hearing thresholds were determined using auditory brainstem response measurements 1 day prior to the first kanamycin or saline injection. Hearing loss in mice continues to worsen until 3 weeks after the end of the aminoglycoside treatment, at which time the degree of hearing loss stabilizes (Wu et al. 2001). Therefore, posttest ABR thresholds were obtained 3 weeks after the last kanamycin or saline injection. Threshold shifts (i.e., posttest threshold minus pretest threshold) were calculated for each mouse at all six frequencies tested. Figure 1 shows mean threshold shifts for each experimental group. Saline-injected mice of both genotypes (wild type and Hsp70 overexpressing) had no significant hearing loss at any frequency tested (4 kHz: $F_{1,17}=0.25$ and $p>0.05$; 8 kHz: $F_{1,17}=0.01$ and $p>0.05$; 11.3 kHz: $F_{1,17}=0.07$ and $p>0.05$; 16 kHz: $F_{1,17}=0.07$ and $p>0.05$; 22.4 kHz: $F_{1,17}=0.36$ and $p>0.05$; 32 kHz: $F_{1,17}=0.00$ and $p>0.05$; Fig. 1). Kanamycin-treated wild-type mice showed significant threshold shifts at all frequencies tested, with larger threshold shifts at higher frequencies (4 kHz: $F_{1,17}=8.39$ and $p<0.05$; 8 kHz: $F_{1,17}=11.83$, $p<0.05$; 11.3 kHz: $F_{1,17}=9.41$, $p<0.05$; 16 kHz: $F_{1,17}=19.89$, $p<0.05$; 22.4 kHz: $F_{1,17}=4.75$, $p<$

Fig. 1 Hsp70 overexpression inhibits kanamycin-induced hearing loss. Hsp70-overexpressing mice and their wild-type littermates underwent hearing testing via auditory brainstem response measurement prior to receiving kanamycin or saline. Three weeks after the end of the treatment, hearing testing was repeated. Threshold shift is reported as the difference between pretest and posttest ABR thresholds. Saline-injected Hsp70-overexpressing mice (closed square) and their saline-injected wild-type littermates (closed triangle) had no significant hearing losses. Wild-type animals that received kanamycin (open triangle) had significant hearing losses at all frequencies tested, while Hsp70-overexpressing mice were protected against kanamycin-induced hearing loss (open square). Hsp70 OE Hsp70-overexpressing mice. Data points are means $\pm$ standard error of the mean for $n=5-6$ animals per group. Asterisks indicate a significant difference between wild-type and Hsp70-overexpressing mice in the kanamycin-treated condition (two-way ANOVA $p<0.05$)

0.05; 32 kHz: $F_{1,17}=5.11$, $p<0.05$; Fig. 1). In agreement with previous reports, there was considerable variability in the sizes of the threshold shifts among kanamycin-injected wild-type animals (Wu et al. 2001). One kanamycin-treated wild-type mouse was profoundly hearing-impaired (i.e., no response at the output limits of the loudspeaker) at all frequencies tested. Three out of five were profoundly hearing-impaired at 22.4 kHz, and all five kanamycin-treated wild-type mice had profound hearing losses at 32 kHz. In contrast, threshold shifts of kanamycin-treated Hsp70-overexpressing mice were significantly smaller than those of kanamycin-treated wild-type mice at all frequencies tested (4 kHz: $F_{1,17}=8.39$, $p<0.05$; 8 kHz: $F_{1,17}=11.84$, $p<0.005$; 11.3 kHz: $F_{1,17}=9.41$, $p<0.01$; 16 kHz: $F_{1,17}=19.89$, $p<0.001$; 22.4 kHz: $F_{1,17}=4.75$, $p<0.05$; 32 kHz: $F_{1,17}=5.11$, $p<0.05$; Fig. 1). These data indicate that Hsp70 overexpression inhibits aminoglycoside-induced hearing loss.

Figure 2 shows representative posttest ABR recordings at 16 kHz from each group. Saline-injected wild-type (Fig. 2a) and Hsp70-overexpressing mice (Fig. 2b) showed normal auditory brainstem responses with five to seven distinct peaks at approximately 1-ms intervals. As stimulus intensity decreased, each waveform decreased in amplitude and increased in latency until the waveform was not discernable at threshold. Following kanamycin injections, wild-type mice showed severe hearing loss, represented in Fig. 2c by the absence of a discernible waveform at the output limits of the stimulus generating equipment. In contrast, kanamycin-injected Hsp70-overexpressing mice retained normal ABR waveforms (Fig. 2d).

Hsp70 overexpression inhibits aminoglycoside-induced cochlear hair cell death in vivo

To determine whether Hsp70 inhibits aminoglycoside-induced hair cell death in vivo, cochleae were prepared for immunohistochemistry using antimyosin VIIa as a hair cell marker (Fig. 3). Saline-treated wild-type (Fig. 3a, e, i) and saline-treated Hsp70-overexpressing (Fig. 3b, f, j) animals showed normal arrangements of three rows of outer hair cells and a single row of inner hair cells in all three cochlear turns (base, middle, and apex). Outer hair cells are more susceptible to aminoglycoside-induced death than inner hair cells (Forge and Schacht 2000). Inner hair cells were intact in saline-injected mice of both genotypes and in kanamycin-injected Hsp70-overexpressing mice. In kanamycin-treated wild-type mice (Fig. 3c, g, k), most inner hair cells remained, with only occasional inner hair cell loss in the basal and middle turns. Saline-injected Hsp70-overexpressing (Fig. 3b, f, j) and wild-type mice (Fig. 3a, e, i) showed little evidence of outer hair cell loss, with only occasional missing outer hair cells. In contrast, outer hair cell loss in kanamycin-treated wild-type mice was severe in the basal turn (Fig. 3k), with all outer hair

Fig. 2 ABR thresholds following kanamycin treatment. Shown are representative posttest ABR recordings at 16 kHz recorded 3 weeks after the last day of injections. The recordings shown are from an individual mouse in each of the four treatment groups: **a** Wild-type mouse, saline-injected; **b** Hsp70-overexpressing mouse, saline-injected; **c** wild-type mouse, kanamycin-injected; and **d** Hsp70-overexpressing mouse, kanamycin-injected. At this frequency, ABRs of two (out of five) kanamycin-treated wild-type mice (**c**) were undetectable. In contrast, kanamycin-treated Hsp70-overexpressing mice (**d**) retained normal ABR waveforms. Arrowheads denote ABR threshold. NR denotes no response at output limits of equipment ("threshold" assigned=105 dB SPL)

cells missing. There was considerable variability in the amount of outer hair cell loss in the middle turns of kanamycin-treated wild-type mice (Fig. 3g), with outer hair cell losses ranging from moderate in some animals to severe in others. In the apical turn, kanamycin-injected wild-type mice showed only scattered loss of outer hair cells (Fig. 3c). In contrast, most outer hair cells were intact in the apical (Fig. 3d), middle (Fig. 3h), and basal (Fig. 3i) turns of cochleae from Hsp70-overexpressing mice treated with kanamycin. Only occasional outer hair cells were missing. These data indicate that Hsp70 has a robust protective effect against aminoglycoside-induced cochlear hair cell death in vivo.

Kanamycin in the vestibular system

In addition to cochleae, utricles were harvested from kanamycin-treated Hsp70-overexpressing mice and their

wild-type littermates 3 weeks after the completion of the drug treatment. Hair cell densities did not vary significantly in utricles from kanamycin-treated Hsp70-overexpressing mice and their wild-type littermates in either the striolar or extrastriolar regions (Fig. 4). In addition, utricular hair cells were not killed by the aminoglycoside treatment since both extrastriolar and striolar hair cell densities were comparable to those of untreated control utricles (striola: $F_{2,11}=0.93$, $p>0.05$; extrastriola: $F_{2,15}=1.32$, $p>0.05$). These data indicate that the kanamycin treatment was cochleotoxic but not vestibulotoxic.

Discussion

Stress-induced Hsp induction can promote cellular survival in a number of systems. We have previously demonstrated that both heat shock and Hsp70 overexpression inhibit aminoglycoside-induced vestibular hair cell death in organ culture (Cunningham and Brandon 2006; Taleb et al. 2008). Here, we have extended our in vitro studies of mouse utricle to in vivo studies of mouse cochlea and hearing function. Our data indicate that Hsp70 has a robust protective effect against aminoglycoside-induced hearing loss and cochlear hair cell death in vivo.

Heat shock results in robust induction of Hsp70 in adult mouse utricles in vitro (Cunningham and Brandon 2006; Taleb et al. 2008). In that preparation, heat shock is protective against aminoglycoside-induced hair cell death at a range of aminoglycoside doses (Cunningham and Brandon 2006; Taleb et al. 2008). Because heat shock results in upregulation of a number of proteins and because Hsp70 is the most highly induced Hsp, we previously examined whether Hsp70 alone could inhibit aminoglycoside-induced hair cell death in an organ culture model system of the mouse utricle (Taleb et al. 2008). Utricles from mice that constitutively overexpress Hsp70 were protected from aminoglycoside-induced hair cell death in vitro (Taleb et al. 2008). The protective effect of Hsp70 against aminoglycoside-induced hair cell death was observed at a range of neomycin concentrations, suggesting that induction of Hsp70 may hold the potential to be developed into a relevant clinical cotherapy for patients receiving aminoglycoside antibiotics. To advance these studies toward the goal of developing a therapy aimed at preventing the ototoxic effects of aminoglycosides, we have here examined the protective effect of Hsp70 against aminoglycoside-induced hair cell death and hearing loss in the whole animal. To determine whether Hsp70 is protective against aminoglycoside-induced hearing loss and hair cell death in vivo, we utilized mice that constitutively overexpress Hsp70 (Marber et al. 1995). Compared to wild-type mice, these mice are more resistant

Fig. 3 Hsp70 overexpression inhibits kanamycin-induced cochlear hair cell death in vivo. After ABR posttesting, mice were euthanized, and their cochleae were prepared for immunohistochemistry using antimyosin VIIa to label hair cells. Saline-injected wild-type (**a**, **e**, **i**) and Hsp70-overexpressing mice (**b**, **f**, **j**) showed normal cochlear morphology with a single row of inner hair cells (IHCs) and three rows of outer hair cells (OHCs) intact in the apical (**a**, **b**), middle (**e**, **f**), and basal (**i**, **j**) cochlear turns. In kanamycin-injected wild-type mice, most outer hair cells in the basal turn were missing (**k**).

Significant outer hair cell loss was also observed in middle turns of kanamycin-treated wild-type mice (**g**). Only occasional outer hair cells were missing in the apical turns of kanamycin-treated wild-type mice (**c**). Inner hair cells remained largely intact in all three turns of kanamycin-treated wild-type mice (**c**, **g**, **k**). Kanamycin-injected Hsp70-overexpressing mice (**d**, **h**, **l**) showed intact outer and inner hair cells, with only occasional outer hair cells missing. OHC1–3 outer hair cells rows 1–3, IHC inner hair cells, Hsp70 OE Hsp70-overexpressing mice. Scale bar in **l**=25 μ m and applies to all panels

to ischemia in both the heart and brain (Marber et al. 1995; Tsuchiya et al. 2003; McArdle et al. 2004). Hsp70-overexpressing mice and their wild-type littermates were treated with an ototoxic dose of the aminoglycoside antibiotic kanamycin for 2 weeks. Our data indicate that Hsp70 provides a robust protective effect against aminoglycoside-induced hearing loss. In addition, Hsp70 overexpression significantly inhibited kanamycin-induced cochlear hair cell death in vivo.

There are two types of hair cells in the cochlea, and they demonstrate differential sensitivity to aminoglycoside-induced damage (Forge and Schacht 2000). Outer hair cells are known to be more sensitive to aminoglycoside-induced death than inner hair cells, and the results of the current study support this finding: even in areas with severe loss of outer hair cells, most inner hair cells remained intact. In addition to the differential sensitivities of the two hair cell types, it is also well known that there are spatial differences in sensitivity to aminoglycosides, with outer hair cells of the basal turn of the cochlea being significantly more sensitive to aminoglycoside-induced damage than those of the apical turn (Forge and Schacht

2000). It has been suggested that this spatial difference in sensitivity may be due to intrinsic differences in antioxidant capacity in apical vs. basal hair cells (Sha et al. 2001). In agreement with many previous reports, we found severe loss of outer hair cells in the basal turns of kanamycin-treated wild-type mice, with little damage to outer hair cells in the apical turns of the same mice. These spatial differences in sensitivity correlate with the data on aminoglycoside-induced hearing loss. Since basal hair cells detect high-frequency sounds and apical hair cells detect lower-frequency sounds, aminoglycoside-induced hearing loss is most severe in the higher frequencies. Our data show that Hsp70 overexpression protects outer hair cells in the basal and middle turns of the cochlea. In addition, Hsp70 overexpression inhibits kanamycin-induced hearing loss. Because some of the kanamycin-injected wild-type animals had no ABR responses at the output limits of the equipment in the higher frequencies, our data probably underestimate the protective effect of Hsp70 overexpression at these frequencies.

We previously reported that utricles from Hsp70-overexpressing mice are protected against aminoglycoside-

Fig. 4 Kanamycin did not result in vestibular hair cell death *in vivo*. Control utricles were dissected from wild-type mice (**a**) and Hsp70-overexpressing littermates (**c**), immediately fixed and double-labeled for calmodulin and calbindin. Utricles from kanamycin-injected wild-type (**b**) and Hsp70-overexpressing (**d**) mice were collected 3 weeks after the last kanamycin injection. Extrastriolar and striolar hair cell densities (**e**) were not significantly different in any condition (one-way ANOVA $p>0.05$). Scale bar in **d** represents 20 μm and applies to panels **a–d**. Bars represent mean hair cell densities $\pm$ standard error of the mean for $n=4$ –12 utricles per condition

induced hair cell death *in vitro* (Taleb et al. 2008). Therefore, we examined the utricles from the kanamycin-injected mice in the current study. We found that systemic kanamycin did not result in hair cell death in the utricle. Aminoglycosides exhibit organ preferences, and some aminoglycosides are more toxic to the cochlea, while others are more toxic to the vestibular organs (Forge and Schacht 2000). Kanamycin has been previously reported to be more cochleotoxic than vestibulotoxic (Aran 1995; Taylor et al. 2008). Our data support the selective cochleotoxicity of kanamycin. We found that in wild-type mice, kanamycin treatment leads to significant hair cell death in the cochlea (Fig. 3), while the utricle shows no evidence of hair cell loss (Fig. 4). This finding is in contrast with a previous report indicating that hair cells were often replaced by expanding supporting cells in utricles from kanamycin-treated mice (Wu et al. 2001). The kanamycin-induced

vestibulotoxicity was observed in CBA and BALB/c mice, while C57BL/6 mice were reported to be more resistant to aminoglycoside-induced vestibular damage (Wu et al. 2001). Hsp70-overexpressing mice are on a mixed BALB/c $\times$ C57BL/6 genetic background, suggesting that background strain differences may account for the difference between our findings and those of Wu et al. (2001). In addition, our studies utilized a lower dose of kanamycin (2×700 mg/kg/day) than the dose reported by Wu et al. (2001) to result in utricular hair cell death (2×900 mg/kg/day).

The protective effects of both JNK inhibitors and caspase inhibitors against aminoglycoside-induced ototoxicity have been demonstrated *in vivo* (Pirvola et al. 2000; Matsui et al. 2003). Besides being important regulators of pathophysiological conditions such as ototoxicity, both JNK and caspases are regulators of physiological processes, such as apoptosis (Earnshaw et al. 1999; Chen and Tan

2000). Due to their involvement in physiological processes, systemic inhibition of caspase activity or JNK signaling would likely interfere with the homeostasis of the organism. Thus it seems unlikely that systemic caspase inhibitors or JNK inhibitors will be developed into safe and viable cotherapies aimed at inhibiting aminoglycoside-induced ototoxicity. However, it is possible to safely induce Hsp70 in the whole organism, either by experimental manipulation (heat stress) (Dechesne et al. 1992; Yoshida et al. 1999; Sugahara et al. 2003) or by administration of a therapeutic agent (Hirakawa et al. 1996; Ooie et al. 2001; Oda et al. 2002; Mikuriya et al. 2005; Sone et al. 2005). In order to begin to develop Hsp70 as an otoprotective therapy against aminoglycoside-induced hearing loss, a safe method of Hsp70 induction is required. Total body heat shock induces Hsp70 in guinea pig and mouse cochleae (Dechesne et al. 1992; Yoshida et al. 1999; Sugahara et al. 2003). In addition, local heat shock can induce Hsp70 in the inner ear (Sugahara et al. 2003). Both total body and local heat shock inhibit noise-induced hearing loss and hair cell death in vivo (Yoshida et al. 1999; Sugahara et al. 2003). A potentially appealing clinical alternative to heat shock is pharmaceutical induction of Hsp70. One pharmacological inducer of Hsps is geranylgeranylacetone (GGA). GGA has been safely used in Japan in the clinical treatment of gastric ulcers (Shirakabe et al. 1995; Nagasawa et al. 1998; Miyake et al. 2004; Qian et al. 2007). GGA has been shown to induce Hsp70 in variety of tissues, including the gastric mucosa, heart, liver, brain, and cochlea (Hirakawa et al. 1996; Ooie et al. 2001; Oda et al. 2002; Mikuriya et al. 2005; Sone et al. 2005). Recent in vitro studies have suggested a protective effect of GGA against aminoglycoside-induced ototoxicity (Takumida and Anniko 2005; Sano et al. 2007). Another pharmacological inducer of Hsps is celastrol, a triterpene extract that induces Hsps via Hsf1 activation in a variety of cell types (Westerheide et al. 2004; Zhang and Sarge 2007; Trott et al. 2008). Thus, pharmacological induction of Hsps may hold potential for development into a protective cotherapy against aminoglycoside-induced ototoxicity.

Here, we present evidence that Hsp70-mediated protection against aminoglycoside-induced hair cell death in vitro translates into the whole animal. Hsp70 overexpression resulted in significant protection against aminoglycoside-induced cochlear hair cell death in vivo. In addition, Hsp70 significantly inhibited aminoglycoside-induced hearing loss. These data suggest that Hsp70 induction may represent a potential therapeutic approach for the prevention of aminoglycoside-induced hearing loss.

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