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Inhibition of the c-Jun N-Terminal Kinase-Mediated Mitochondrial Cell Death Pathway Restores Auditory Function in Sound-Exposed Animals

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ABSTRACT

We tested and characterized the therapeutic value of round window membrane-delivered (RWM) D-JNKI-1 peptide (Bonny et al., 2001) against sound trauma-induced hearing loss. Morphological characteristics of sound-damaged hair cell nuclei labeled by Hoechst staining show that apoptosis is the predominant mode of cell death after sound trauma. Analysis of the events occurring after sound trauma demonstrates that c-Jun N-terminal kinase (JNK)/stress-activated protein kinase activates a mitochondrial cell death pathway (i.e., activation of Bax, release of cytochrome *c*, activation of procaspases, and cleavage of fodrin). Fluorescein isothiocyanate (FITC)-conjugated D-JNKI-1 peptide applied onto an intact cochlear RWM diffuses through this membrane and penetrates cochlear tissues with the exception of the stria vascularis. A time sequence of fluorescence measurements demonstrates that FITC-labeled D-JNKI-1 remains in cochlear tissues for as long as 3 weeks. In addition to blocking JNK-mediated activation of a mitochondrial cell death pathway, RWM-delivered D-JNKI-1 prevents hair cell death and development of a permanent shift in hearing threshold that is caused by sound trauma in a dose-dependent manner (EC_{50} = 2.05 μM). The therapeutic window for protection of the cochlea from sound trauma with RWM delivery of D-JNKI-1 extended out to 12 h after sound exposure. These results show that the mitogen-activated protein kinase/JNK signaling pathway plays a crucial role in sound trauma-initiated hair cell death. Blocking this signaling pathway with RWM delivery of D-JNKI-1 may have significant therapeutic value as a therapeutic intervention to protect the human cochlea from the effects of sound trauma.

The c-Jun N-terminal kinase (JNK)/stress-activated protein kinase, a member of the mitogen-activated protein kinase (MAPK) family, can be activated by a variety of environmental stresses, such as UV irradiation or exposure to toxins (Derijard et al., 1994; Kyriakis et al., 1994). JNKs can phosphorylate a variety of cytoplasmic and nuclear proteins, including c-Jun (Hibi et al., 1993; Mielke and Herdegen, 2000), activating transcription factor 2 (Gupta et al., 1995), and ETS-containing factors (Whitmarsh et al., 1995). In the cochlea, JNK can be activated by various forms of insults such as loss of trophic factor support (Scarpidis et al., 2003), drug ototoxicity (Ylikoski et al., 2002; Wang et al., 2003) and sound exposure (Pirvola et al., 2000; Wang et al., 2003). Blocking the MAPK/JNK cell death signal pathway with antisense oligonucleotides that target the downstream target of phosphorylated JNK (i.e., c-Jun) can prevent the death of auditory neurons in vitro (Scarpidis et al., 2003). The peptide inhibitor CEP-1347 (derived from indolecarbazole, K252a), which blocks the MAPK/JNK cascade at the level of mixed lineage kinases (Maroney et al., 2001), can prevent apoptosis of hair cells in organ of Corti explants exposed to an ototoxic level of neomycin and protect auditory neurons from stress-initiated death in dissociated ganglion cell cultures after loss of trophic factor support (Pirvola et al., 2000). Systemic ad-

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ABBREVIATIONS: JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; CAP, compound action potential; SEM, scanning electron microscopy; IHC, inner hair cell; OHC, outer hair cell; PTS, permanent threshold shift; HA, hyaluronic acid; RWM, round window membrane; FITC, fluorescein isothiocyanate; AP, artificial perilymph solution; PBS, phosphate-buffered saline; SPL, sound pressure level; RT, room temperature.

ministration of CEP-1347 provides partial protection against hearing loss induced by sound trauma (Pirvola et al., 2000). Consistent with these observations, treatment of neomycin-exposed organ of Corti explants with D-JNKI-1, a chemically synthesized cell-permeable JNK ligand that blocks JNK-activation of c-Jun (Borsello and Bonny, 2004), prevents phosphorylation of c-Jun and protects the sensory hair cells (Wang et al., 2003). Perilymphatic perfusion of D-JNKI-1 in vivo resulted in almost complete protection of the cochlea from neomycin-induced hair cell and hearing loss (Wang et al., 2003). In another study, the MAPK/JNK signaling pathway was activated in response to cisplatin toxicity, but in this instance, perilymphatic infusion of D-JNKI-1 did not protect against cisplatin ototoxicity; rather, it potentiated cisplatin's ototoxicity (Wang et al., 2004). In contrast, protection of both auditory hair cells and auditory function was observed with the D-JNKI-1 peptide in sound trauma-exposed animals (Wang et al., 2003). The specific downstream targets of MAPK/JNK signal-mediated cochlear hair cell death after sound trauma are unknown at present. Another problem is that to date, there have been no studies that examine the efficacy of D-JNKI-1 treatment using a protocol that would be compatible with clinical applications (e.g., delivery onto the RWM of the cochlea and not infusion into the scala tympani via a cochleostomy). Finally, no data have been acquired concerning D-JNKI-1 treatment after the initial sound trauma exposure, which is important information for clinicians in their search for a treatment to rescue hearing after sound exposure has occurred.

The present study was designed to identify the downstream targets of MAPK/JNK signal-mediated hair cell death and the therapeutic value of protection of hearing function when D-JNKI-1 is applied onto the RWM not only before but also after exposure to a sound trauma.

Materials and Methods

Pigmented guinea pigs (250–300 g; Charles River, L'Arbresle, France) were used in this study. The care and use of animals followed the animal welfare guidelines of the Institut National de la Santé et de la Recherche Medicale under the approval of the French Ministère de l'Agriculture et de la Forêt. All efforts were made to minimize the number of animals used and any suffering caused by the experimental protocols. Table 1 summarizes the experimental protocol and details the number of animals used for each protocol.

Drug Preparation

Artificial perilymph solution (AP) consisted of 137 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $NaHCO_3$, and 11 mM glucose, pH 7.4, with osmolarity of 304 ± 4.3 mOsm/kg. A 20 mM stock solution of D-JNKI-1 (Auris Medical AG, Lohn-Ammannsegg, under license from Xigen S.A., Lausanne, both Switzerland), 1 mM stock solution of the D form of TAT peptide (i.e., "TAT-empty") and the inactive mutant forms of JNKI-1 (i.e., JNKI-1-mut), and the FITC-conjugated D-JNKI-1 (Xigen S.A., Lausanne, Switzerland) were prepared in 0.1 M PBS at pH 7.2.

Before each experiment, the D-JNKI-1 solution was diluted in AP to a final concentration of either 0.01, 0.1, 1, 3.3, 10, 33, or 100 μM. JNKI-1-mut, TAT-empty, and the FITC-conjugated D-JNKI-1 were diluted in AP to a final concentration of 100 μM.

Round Window Membrane Drug Delivery

Thirty-Minute RWM Delivery. To ensure that delivery onto the RWM allowed D-JNKI-1 to reach the sensory epithelium, we gently

TABLE 1
Experimental protocol

Experiments	Methods	Markers/Drugs and Time Course[a]	No. of Animals
Sound-induced JNK-mediated mitochondrial cell death pathway			
Nature and time course of cell death	Immunocytochemistry	Calbindin, neurofilament, Hoechst	32
	TEM		6
Cell death mechanisms	Western blots	p-c-Jun	28
	Immunocytochemistry	Bax, fodrine, cytochrome *c*, cytochrome *c*-Bax-Hoechst cleaved fodrin-calbindin-Hoechst	18
Protective effects of D-JNKI-1 against sound trauma			
Diffusion and penetration	Confocal imaging	100 μM FITC-D-JNKI-1	23
Control	Functional assessment	100 μM TAT-empty, 100 μM JNK-mut	6 (3 per drugs)
Protective effects	Dose-response	0.01–100 μM D-JNKI-1	42 (6 per doses)
	SEM	100 μM D-JNKI-1	6 (3 sound exposed untreated + 3 sound-exposed treated)
Therapeutic window	Time-response (minipump)	30 min before and 30 min, 1 h, 4 h, 6 h, 12 h, 24 h after sound exposure.	42 (6 per time point)
	Time-response (HA gel)	30 min before and 30 min, 4 h, 12 h after sound exposure	24 (6 per time point)
	Western blots	10 μM D-JNKI-1, 100 μM D-JNKI-1, 100 μM JNK-mut	16 (4 per drugs and per time points)
	Immunocytochemistry	100 μM of D-JNKI-1	6

TEM, transmission electron microscopy.
[a] Markers corresponds to the sound-induced protocol, and Drugs and Time Course corresponds to the protective effects protocol.

(3 μl/min) infused the RWM niche with 100 μM concentration of FITC-conjugated D-JNKI-1 over the course of 30 min using a glass pipette. The contralateral cochlea served as a control.

Seven-Day RWM Delivery Via an Osmotic Minipump. Before surgery, the Alzet 2001 minipump (DURECT Corporation, Cupertino, CA) was filled with 200 μl of AP containing 0.01 to 100 μM concentration of D-JNKI-1, 100 μM of TAT-empty, or 100 μM of JNKI-1-mut under sterile conditions. A glass micropipette was connected to the PE50 polyethylene tubing that connected to the osmotic pump. The pipette and the tubing were then filled and connected to the flow moderator on the pump. The minipump flow rate was 1 μl/h.

Any protective effect of D-JNKI-1 against sound trauma was tested by Western blots, immunocytochemistry, scanning electron microscopy, and functional evaluation. In all cases, D-JNKI-1 was applied onto the RWM via an osmotic minipump for 7 days, and each animal was implanted with a minipump 2 days before sound exposure. Furthermore, JNKI-1-mut and TAT-empty were used as negative controls to demonstrate the specificity of the peptide inhibitor.

We also evaluated the ability of D-JNKI-1 to rescue cochlear function from sound-induced hearing when applied after the initial exposure to the sound trauma. Here, the 7-day minipump containing D-JNKI-1 was implanted 30 min before or 30 min, 1 h, 4 h, 6 h, 12 h, or 24 h after the initial exposure to the sound trauma.

RWM Delivery of D-JNKI-1 via a Hyaluronic Acid Gel. Additional groups of animals were used to test the efficiency of D-JNKI-1 when applied after sound trauma using a hyaluronic acid (HA; molecular mass, 2.4×10^6 Da; Genzyme Advanced Biomaterials, Cambridge, MA) gel. A final concentration of 100 μM D-JNKI-1 was freshly prepared in a 2.6% HA gel before each experiment. The HA gel containing D-JNKI-1 was placed directly onto the RWM.

Surgery

Experiments were designed to record the compound action potentials (CAPs) in awake animals from a connector plug fixed on the head of the animal during minipump or HA gel implantation. This method has been extensively described elsewhere (Wang et al., 2002). In brief, animals were anesthetized with an intraperitoneal injection of 6% sodium pentobarbital (Sanofi, Montpellier, France; dose, 0.3 ml/kg). Each bulla was opened under sterile conditions. The recording electrode was placed on the bony edge of the RWM, leaving enough space to appose the infusion microcatheter (tip diameter, 0.35 mm) or 2 μl of HA gel onto the RWM under microscopic control.

Functional Assessment

CAPs of the auditory nerve were elicited by tone bursts of alternating polarity (1-ms rise/fall, 8-ms duration) applied to the ear at a rate of 10/s from 0 to 100 dB SPL in 5-dB steps in a free field via a JBL 075 earphone. Cochlear responses were amplified (gain, 2000), averaged (128 samples), and stored on a Pentium personal computer operating at 100 MHz (Dimension; Dell Computer Corporation, Round Rock, TX). CAP recordings were measured peak-to-peak between the negative depression N1 and the subsequent positive wave P1. The threshold of the CAP was defined as the intensity in decibel SPL needed to elicit a measurable response (≥5 μV).

Sound Trauma

The animals were then exposed to sound trauma (130-dB SPL for 15 min) under light anesthesia (0.2 ml/kg of pentobarbital). Sound trauma was induced by a continuous 6-kHz pure tone generated by a wave form synthesizer (Hewlett Packard 8904A; Hewlett Packard, Palo Alto, CA), which was routed through a programmable attenuator and presented to the ears in free field via a JBL 075 earphone positioned 10 cm in front of the animal's head. The sound level was measured using a calibrated half-inch microphone (model 4314; Brüel & Kjaer GmbH, Bremen, Germany) and a calibrating amplifier (model 2606; Brüel & Kjaer GmbH).

Morphological Assessment

Confocal Microscopy. Animals were killed with an overdose of pentobarbital. The cochleae were then rapidly removed and perfused with 4% paraformaldehyde in 0.1 M PBS, pH 7.3, postfixed for 2 h in the same fixative at room temperature (RT), and rinsed in PBS. The surface of the organ of Corti was exposed by removing the otic capsule, stria vascularis, tectorial, and Reissner's membranes. For all experiments, 10 μm thicknesses of cochlea cryostat sections were prepared after microdissection, except for FITC fluorescence intensity evaluation of the cochleae infused with 100 μM concentration of FITC-conjugated D-JNKI-1, in which the cryostat sections were performed after decalcification of the cochleae with 10% of EDTA in PBS for 2 days at 4°C. All of the observations were performed on a Leica DMRB microscope (blocks of filters N2.1 and A4; Leica, Wetzlar, Germany).

Sensory Hair Cells and Nerve Endings. The assessment of hair cell and nerve ending integrity was performed on surface preparations from nontreated cochleae before and 1 h, 6 h, 12 h, 24 h, 48 h, 5 days, and 15 days after sound trauma. The immunostaining was performed with two primary antibodies [i.e., a rabbit polyclonal anti-calbindin-D-28K antibody (1:2000 dilution; Sigma, France) to label the sensory hair cells and a mouse monoclonal antibody to NF 200 kDa (clone, JG1; 1:100; Affinity BioReagents, Golden, CO)] to label the auditory nerve endings. Secondary antibodies were an Alexa 568-labeled goat anti-rabbit antibody together with an Alexa 488-labeled goat anti-mouse antibody (1:500; Invitrogen, Carlsbad, CA).

Nuclear Morphology. Nuclear morphology of the cochlear cells was studied on surface preparations by staining the chromatin of the nuclei with Hoechst 33342 [0.002% (w/v); Sigma Chemical, St. Louis, MO] at RT for 10 min.

Cytochrome *c* and Bax. Cytochrome *c* was detected in cryostat sections with a mouse anti-cytochrome *c* monoclonal antibody (1:200 dilution; BD Biosciences PharMingen, San Diego, CA). The secondary antibody was an Alexa 488-labeled goat anti-mouse antibody (1:500; Invitrogen). Bax was detected with rabbit polyclonal antibody to the Bax (1:750 BD Biosciences PharMingen) and an Alexa 568-labeled secondary antibody (1:1000 dilution, goat anti-rabbit IgG antibody; Invitrogen). The sections were then counterstained with Hoechst 33342 [0.002% (w/v); Sigma Chemical, St. Louis, MO] at RT for 10 min for staining DNA. All rinses were performed with PBS.

Fodrin Cleaved by Caspases. Cleavage of fodrin was detected in cryostat sections with two primary antibodies: a monoclonal antibody against calbindin (1:600 dilution; Sigma) to identify hair cells, and a rabbit polyclonal antibody against cleaved α-fodrin (Asp1185). This latter antibody recognizes only fodrin fragments that have been cleaved by caspases (1:400; Cell Signaling, San Diego, CA). Secondary antibodies were an Alexa 488-labeled goat anti-rabbit antibody together with an Alexa 568-labeled goat anti-mouse antibody (1:1000; Invitrogen). The sections were then counterstained with Hoechst 33342.

Diffusion and Penetration of FITC-Conjugated D-JNKI-1 Peptides. The assessment of FITC-conjugated D-JNKI-1 peptides uptake by the sensory epithelium of the cochlea was performed on cryostat sections. Images of FITC fluorescence were captured using a Leica DMRB microscope, and the average pixel intensity in individual organ of Corti of all turns was measured using Image J software (http://rsb.info.nih.gov/ij/). FITC fluorescence levels were measured using the histogram function in a 20 × 10-pixel box focused on organs of Corti of all turns. Measurements were repeated nine times for each organ of Corti analyzed.

Scanning Electron Microscopy. Cochleae were fixed with a solution of 0.1 M phosphate buffer, pH 7.4, containing 1% osmic acid and immersed in the same fixative for 1 h, washed in phosphate buffer (0.2 M), and subsequently dehydrated in a graded series of ethanol (30–100%). During the 70% ethanol stage, the bony capsules of each cochlea were dissected, and the stria vascularis and Reiss-

ner's membranes were removed to expose the organ of Corti. Cochleae were then critical point-dried in CO_2, coated with gold-palladium, and observed with a Hitachi S4000 scanning electron microscope (SEM) (Hitachi, Tokyo, Japan).

Quantification of SEM analysis was performed by counting missing hair cells from the apex to the base of each cochlea. The results were expressed as a percentage of remaining hair cells in the single row of inner hair cells (IHCs) and of the three rows of outer hair cells (OHCs) over the entire length of the cochlear duct. In control specimens, the guinea pig cochlea contains approximately 2000 IHCs and 7500 OHCs. For hair cell counts, the whole cochlea was divided into 20 segments (1-mm length of basilar membrane for each segment); each segment was expected to contain approximately 100 IHCs and 375 OHCs. A hair cell was counted as absent when its cuticular plate was missing and it was replaced with a phalangeal scar.

Transmission Electron Microscopy

Animals were decapitated during deep anesthesia, and their cochleae were prepared using our standard protocol for fixation and plastic embedding (Puel et al., 1994). Semithin and ultrathin radial sections of the plastic embedded organ of Corti were cut from the basal and middle turns and observed using a Hitachi 7100 electron microscope (Hitachi) at the Centre de Resources d'Imagerie Cellulaire de Montpellier.

Western Blotting

After tissue homogenization in Laemmli sample buffer, proteins were separated on 10% SDS-polyacrylamide gel electrophoresis and transferred to nylon membranes. Blots were incubated overnight at 4°C with rabbit polyclonal antibody to c-Jun (1:1000), rabbit polyclonal antibody to phospho-Ser73-Jun (1:1000), or rabbit polyclonal antibody to the N-terminal of Bax (1:750; all from Upstate Biotechnology, Lake Placid, NY) that recognizes only the activated form of Bax, or a monoclonal antibody to 240/280 kDa α-fodrin (all spectin) (1:1000; Affiniti Research Products, Devon, UK), or mouse monoclonal antibody to β-actin (1:500; Sigma) followed by incubation with alkaline phosphatase-conjugated secondary antibody (Sigma). Protein-antibody complexes were revealed with the BCIP/NBT-Purple Liquid Substrate (Sigma). Image scans of Western blots were used to quantify phosphorylation levels of c-Jun and expression levels of c-Jun, Bax, or α-fodrin.

Statistics

Statistical analyses were performed using Sigma Plot 2000 for Windows version 6.1 (SPSS Inc., Chicago, IL). All comparisons between means were performed using Student's paired two-tail t tests or a nonparametric Wilcoxon rank test. Results are expressed as means ± S.E.M.

Results

Sound-Induced Hearing Loss and Hair Cell Death

Functional Assessment. In contralateral (left) sound-exposed, untreated cochleae, the average hearing loss measured 20 min after sound trauma reached 65 dB in the higher frequencies of the CAP audiogram, with the maximum temporary hearing loss achieved between the frequencies of 12 and 16 kHz. During the first 2 days after sound exposure, there was a partial recovery in CAP thresholds, leading to a permanent hearing loss of 30 to 40 dB. No further improvement in hearing threshold levels was seen over the remaining 13 days of the experiment (Fig. 1A). These results are consistent with a previous study that used a similar paradigm of sound trauma (Dancer et al., 2001).

Morphology Assessment. Confocal microscopic observations of the surface preparations of the organ of Corti were performed in sound-exposed and nonexposed cochleae from additional animals.

Sensory Hair Cell Loss. Sensory hair cells were considered missing when both calbindin and DNA staining were absent. One hour after sound exposure, a few of the first-row OHCs were missing in the area of maximum sound damage located 14 to 16 mm from the cochlear apex (Fig. 1, B and D). This area corresponds to the region of hair cells that encode for 8 to 14 kHz (Cody and Johnstone, 1980, shows the frequency map in the guinea pig cochlea). Six to 12 hours after sound exposure, some OHCs of all three rows were missing. In contrast, most of the IHCs, the auditory nerve fibers below the IHCs, and the nerve fibers crossing the tunnel to reach the OHCs were still visible. Two days after sound exposure, in the most affected area of the cochlea, most of the OHCs were lost, and the remaining IHCs were damaged extensively (Fig. 2D). By this stage of hair cell degeneration, most of the auditory nerve fibers had disappeared (Fig. 2D). Five days after sound trauma, almost all of the hair cells (both IHCs and OHCs) were missing in the sound-damaged area, and no further hair cell loss was seen over the next 10 days of the experiment (i.e., day 15, Fig. 1D).

Apoptotic and Necrotic Nuclei. To study the nature of the cell death process, we used Hoechst 33342 to stain the cell nuclei. Cell nuclei from non–sound-exposed control cochleae appeared as bright spots with an intense blue color under fluorescent microscopy, indicating uniform staining of their DNA (Fig. $1C_1$). One hour after sound exposure, karyopyknosis changes (characteristic of apoptosis) were observed in the nuclei of some OHCs and IHCs present in the sound-damaged area (Fig. 1, B and $C_{2,3}$). Other nuclei showed characteristic changes of apoptosis such as a shrunken appearance and formation of micronuclei with a greatly increased intensity of Hoechst 33342 fluorescent staining (Fig. 1, C_2 and C_3). Such changes were observed for up to 48 h after trauma. At this stage of hair cell degeneration, there were few but increasing signs of necrosis in the observed patterns of nuclear staining (e.g., enlarged nuclei in the area near the maximally damaged region of the organ of Corti, 13 mm from apex) (Figs. $1C_4$ and 2G).

The time course of hair cell death was determined by counting hair cells in 2-mm length portions around the sound-damaged area (14–16 mm from the apex) in the cochlea at 1 h, 6 h, 12 h, 24 h, 48 h, 5 days, and 15 days after sound trauma. In the control non–sound-exposed cochleae, a 1-mm portion located 15 mm from the apex contains approximately 100 IHCs and 375 OHCs (this hair cell count is referred to as 100% hair cells present) (Fig. 2A). According to Kerr et al. (1972), cells with fragmented and condensed nuclei were counted as apoptotic cells, and those with large and swollen nuclei were counted as necrotic cells. Statistical analysis revealed a significant increase ($p < 0.001$) in apoptotic cell nuclei by 1 h after sound exposure in the area of maximum damage (Fig. 1D). This increase in apoptotic hair cells reached a peak (i.e., 60% of hair cells in the damaged area) 6 h after trauma (Fig. 1D). Two days after sound exposure, the majority of hair cells were missing in the area of maximum damage (Fig. 1D). In the regions directly adjacent to this traumatized zone, there was a slight increase in swollen and enlarged nuclei (i.e., less than 8% of the damaged cells; Fig. 2G), attesting to the occurrence of a necrotic process in these hair cells.

Ultrastructural Analysis. To confirm the occurrence of apoptotic and necrotic processes within the hair cells, ultrastructural analysis was performed 2 days after sound exposure. Compared with hair cells from nonexposed control organ of Corti (Fig. 2, B and C), the hair cells from sound-damaged organ of Corti showed features consistent with apoptosis such as distorted or shrunken cell bodies with electron-dense cytoplasm, fragmentation, and condensation of the nuclear chromatin (Fig. 2, E and F). There were also some hair cells with obvious signs of necrosis such as cell bodies with vacuolated cytoplasm and dispersed cellular debris, and swollen and enlarged nuclei (Fig. 2, H and I). It is interesting that some individual hair cells demonstrated signs typical of both apoptosis (fragmented nucleus and chromatin compaction) and necrosis (cellular debris and disintegrated cytoplasmic membrane; Fig. 2F). Note the undamaged medial efferent endings after sound-induced injury in the OHC, with the only changes being an increased synaptic vesicle density (Fig. 2I).

Mechanisms of Sensory Hair Cell Death

Phosphorylation of c-Jun by JNK and Activation of Bax. Phosphorylation of c-Jun (serine 73) by JNK and activation of Bax were studied by Western blots from control non–sound-exposed cochleae and sound-exposed cochleae at 30 min, 3 h, 6 h, 12 h, 48 h, and 5 days after sound exposure. Compared with non–sound-exposed control cochleae, sound trauma induced a phosphorylation of c-Jun (p-Jun) and a strong activation of Bax. Both events were detected between 30 min and 2 days after trauma, with an apparent peak at 12 h (Fig. 3, A–C), whereas the total levels of c-Jun protein or actin expression did not show any change (Fig. 3, A–C). Application of a 10 or 100 μM concentration of D-JNKI-1 but not 100 μM JNK-I-mut onto the RWM via an osmotic minipump prevented the phosphorylation of c-Jun and the activation of Bax observed 3 and 12 h after sound exposure (Fig. 3, A–C).

Translocation of Bax and Release of Cytochrome *c*. Location of cytochrome *c* was determined by immunostaining cryostat sections. In non–sound-exposed cochleae, immunoreactivity to the anti-cytochrome *c* antibody was localized to the hair cell cytoplasm in a punctate pattern consistent with its mitochondrial localization (Fig. 4A). One hour after sound exposure, immunostaining for cytochrome *c* hair cells was diffuse and distributed uniformly throughout the cell (Fig. 4A). Compared with the pattern of cytochrome staining of non–sound-exposed control cochleae, the intensity of the staining was very weak, with sensory hair cells and supporting cells showing staining barely greater than background level. In contrast, the medial efferents connected to the OHCs still remained strongly labeled, attesting to the specificity of the diffuse cytochrome *c* labeling. This alteration in the pattern of cytochrome *c* staining of the sensory hair cells and supporting cells suggests that cytochrome *c* has been re-

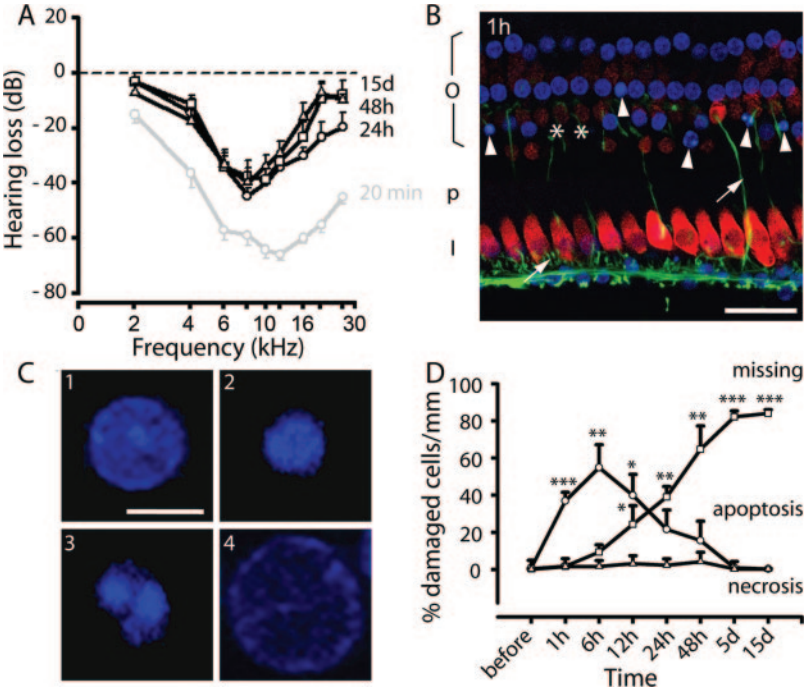

Fig. 1. Sound induces loss of sensory hair cells and hearing threshold. A, the CAP threshold shift corresponds to the difference between the recordings performed before sound trauma and 20 min (●), 24 h (○), 48 h (□), and 15 days (△) after exposure. B, fluorescent microscopic imaging of the maximally damaged region of the organ of Corti in cochleae at 1 h after sound exposure. Surface preparations stained with calbindin (red), NF 200 kDa (green), and nuclear chromatin counterstained blue with Hoechst 33342 dye show that some first row OHCs (O, white asterisks) are missing. Pathological changes within the nuclei of some remaining first and second row OHCs are characterized by dense staining of chromatin fragments and the presence of shrunken, pyknotic nuclei (arrowheads). Nerve fibers (arrows) below the IHCs (I) and OHCs were labeled with anti-NF 200 antibody. C_1 to C_4, different patterns of nuclear chromatin staining with Hoechst 33342. C_1 shows a normal pattern of chromatin staining of a hair cell nucleus from a control non–sound-exposed organ of Corti. C_2 to C_4 show sound-induced changes in the staining of hair cell nuclei: chromatin condensation (C_2), pyknotic nucleus (C_3), and a swollen, enlarged nucleus (C_4). D, time course of appearance of sound-induced apoptotic (○) or necrotic (△) hair cell nuclei and hair cell loss (□) from sound-exposed cochleae. The number of apoptotic, necrotic and missing hair cells is counted in a 2-mm length of maximally damaged cochlear duct from sound-exposed cochleae at eight different time points (before and 1 h, 6 h, 12 h, 24 h, 48 h, 5 days, and 15 days after sound trauma). The results are expressed as the mean percentage of missing hair cells or hair cell nuclei containing apoptotic or necrotic changes ± S.E.M. (bars) for $n = 4$ independent experiments per time point. p, pillar cell. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$. Scale bars: B, 20 μm; C, 4 μm.

leased from the mitochondria into the cytoplasm of damaged cells.

We also examined the activation and subcellular localization of Bax by using an antibody directed to Bax. Organ of Corti from non–sound-exposed control cochleae exhibited a diffuse pattern of weak immunostaining, indicating a cytoplasmic localization of Bax (Fig. 4B). Three hours after trauma, the pattern of Bax immunostaining in the acoustically damaged cochlear cells changed to a dense, punctate pattern consistent with the activation of Bax in the affected sensory cells (Fig. 4B). At the same time, immunostaining for cytochrome *c* was no longer detectable in the sound-damaged cochlear cells. Together, these results suggest that activation of Bax contributed to the release of mitochondrial cytochrome *c* into the cytoplasm. The disappearance of cytochrome *c* staining in the sound-damaged cochlear cells by 3 h after trauma further indicates that, when released into the cytosol, cytochrome *c* is rapidly degraded (Fig. 4B). In addition, 100 μM D-JNKI-1 applied onto the RWM using an osmotic minipump implanted 2 days before sound exposure prevented both sound-induced activation of Bax and release of cytochrome *c* from the mitochondria to the cytoplasm (Fig. 4, A and B).

Cleavage of Fodrin

Expression of Fodrin. The expression of fodrin (all spectin) was measured by Western blots from control non–sound-exposed cochleae and from sound-exposed cochleae at 30 min, 3 h, 6 h, 12 h 48 h, and 5 days after sound exposure. A strong decrease in fodrin expression levels was detected between 30

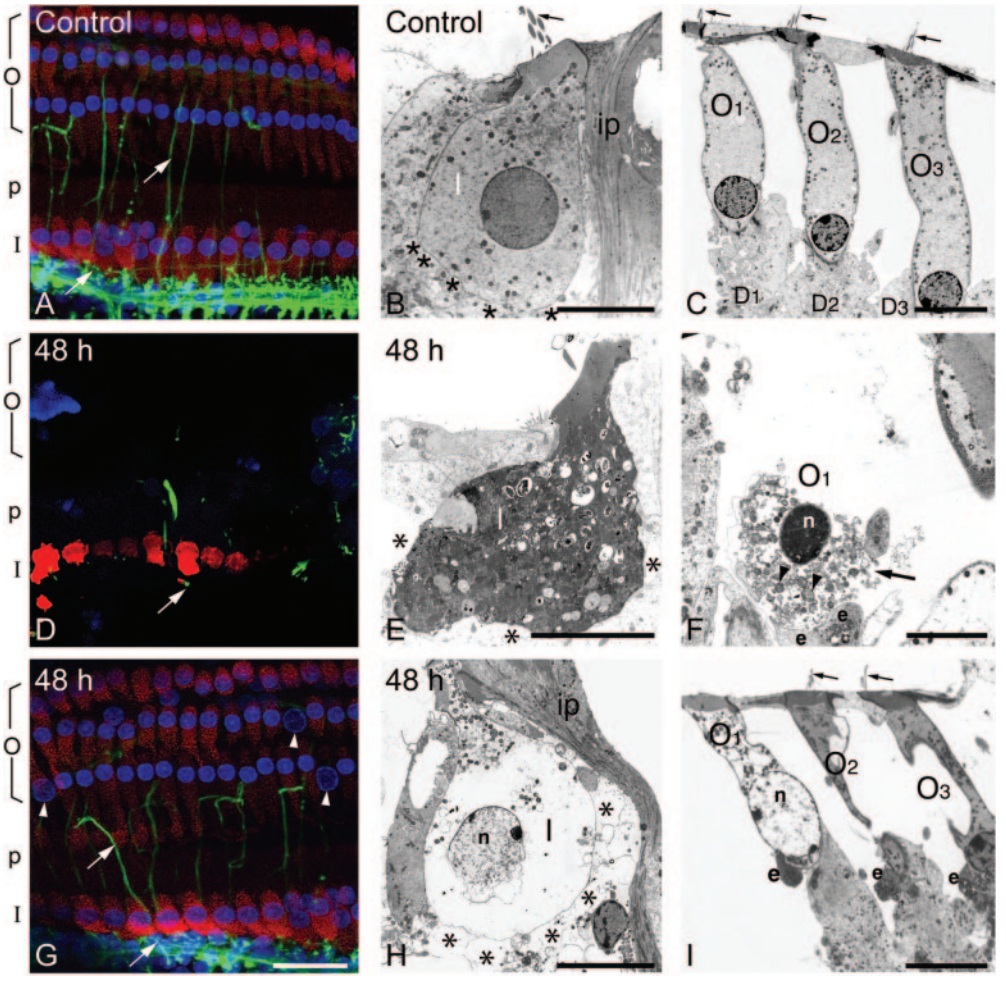

Fig. 2. Nature of sound trauma-induced death of auditory hair cells. A, D, and G, surface preparations of the organ of Corti, stained with calbindin (red) and NF 200 kDa (green), chromatin is counter-stained blue with Hoechst 33342 dye. A, the control non–sound-exposed cochlea. Note the auditory nerve fibers (white arrows) below the IHCs (I) and those crossing the tunnel to reach the OHCs (O). D, in this area of maximal damage (15 mm from apex), there is extensive loss of hair cells from all three OHC rows and from the single row of IHCs with extensive disruption of the auditory nerve fibers (white arrow). G, in an area near the site of maximum damage (13 mm from apex), the hair cell nuclei was swollen and enlarged (white arrowheads), whereas the nerve fibers (arrows) below the IHCs and OHCs are still clearly visible. B, C, E, F, H, and I are transmission electron micrographs. B and C, an IHC (I, in B) and three OHCs (O_1, O_2, and O_3 in C) from control non–sound-exposed animal. Note the IHC, OHCs, and their stereociliary bundles (arrows) and innervation (asterisks in B). E and F, hair cells in the sound-damaged region of the organ of Corti. E, an IHC (I) showing characteristics of apoptosis (i.e., shrinkage of the cell body) and condensation of the cytoplasm with preservation of the cytoplasmic lateral membrane and swollen afferent dendrites (asterisks) at the basal pole of this damaged IHC. F, a degenerating OHC (O_1) with vacuolated cytoplasm, distorted and altered mitochondria (arrowheads), an electron-dense nucleus (n) due to chromatin compaction (signs of an advanced stage of apoptosis), and a disintegrated cytoplasmic membrane (arrow). H and I, images of an area adjacent to the sound-damaged region of the organ of Corti. H, a degenerating IHC (I) showing characteristics of necrosis. I, a first-row OHC (O_1) with vacuolated, swollen cytoplasm and a distended nucleus (n). Note the swollen afferent dendrites (asterisks) at the basal pole of the IHC in H and the normal appearance of stereociliary bundles of the remaining OHCs (O_2 and O_3, arrows) in I. ip, inner pillar cell; D, Deiter cells; e, efferent fibers. Scale bars: A, D, and G, 20 μm; B, C, E, F, H, and I, 10 μm.

min and 5 days, with a maximum level of decrease reached at 3 h after exposure (Fig. 3, A and D). This could be prevented by a RWM of 10 or 100 μM D-JNKI-1 but not 100 μM JNKI-1-mut in AP (Fig. 3, A and D).

Location of Fodrin Cleaved by Activated Caspases. Fodrin is a major component of the cuticular plates of hair cells and a known substrate for effector caspases (e.g., caspase-3). Therefore, we looked for the presence of cleaved fodrin in sound-exposed cochleae by using an antibody that is specific for the 150-kDa N-terminal large fragment of caspase-cleaved fodrin. In our experiments, no cleaved fodrin was detected in the cochlear cells from non–sound-exposed animals (Fig. 4C). Between 6 h and 2 days after sound exposure, there was a marked immunostaining of cleaved fodrin in the region of the cuticular plates, the cytoplasm of hair cells, and in the pillar cells located in the main area of sound damage (Fig. 4C). Other supporting cells from the organ of Corti, cells that compose the stria vascularis, spiral ligament, and spiral ganglion cells did not stain for the presence of cleaved fodrin. Here again, 100 μM D-JNKI-1 applied on the RWM via an osmotic minipump prevented the occurrence of caspase-cleaved fodrin in sound trauma-exposed cochleae (Fig. 4C).

Diffusion and Penetration of FITC-Conjugated D-JNKI-1 Peptides. FITC-conjugated D-JNKI-1 peptides were used to visualize the distribution of this inhibitory peptide to ascertain whether this compound diffused through the RWM and reached cochlear tissues. At 6 h and at 3 days after a 30-min delivery of 100 μM FITC-labeled peptide onto the RWM, FITC-labeled D-JNKI-1 was found to be distributed throughout the scala tympani, reaching the apical region of the cochlea. Distribution of this fluorescent peptide also extended to the scala vestibuli (Fig. 5A). The fluorescent signal showed a base-to-apex gradient pattern of distribution (Fig. 5, A and G) that is consistent with the FITC-labeled peptides being delivered at the base of the cochlea where the RWM is located (Fig. 5A). All of the cochlear cells, including the auditory hair cells and neurons, exhibited fluorescence, indicating that D-JNKI-1 applied onto RWM effectively reached these targeted sensory cells (Fig. 5A). The spiral

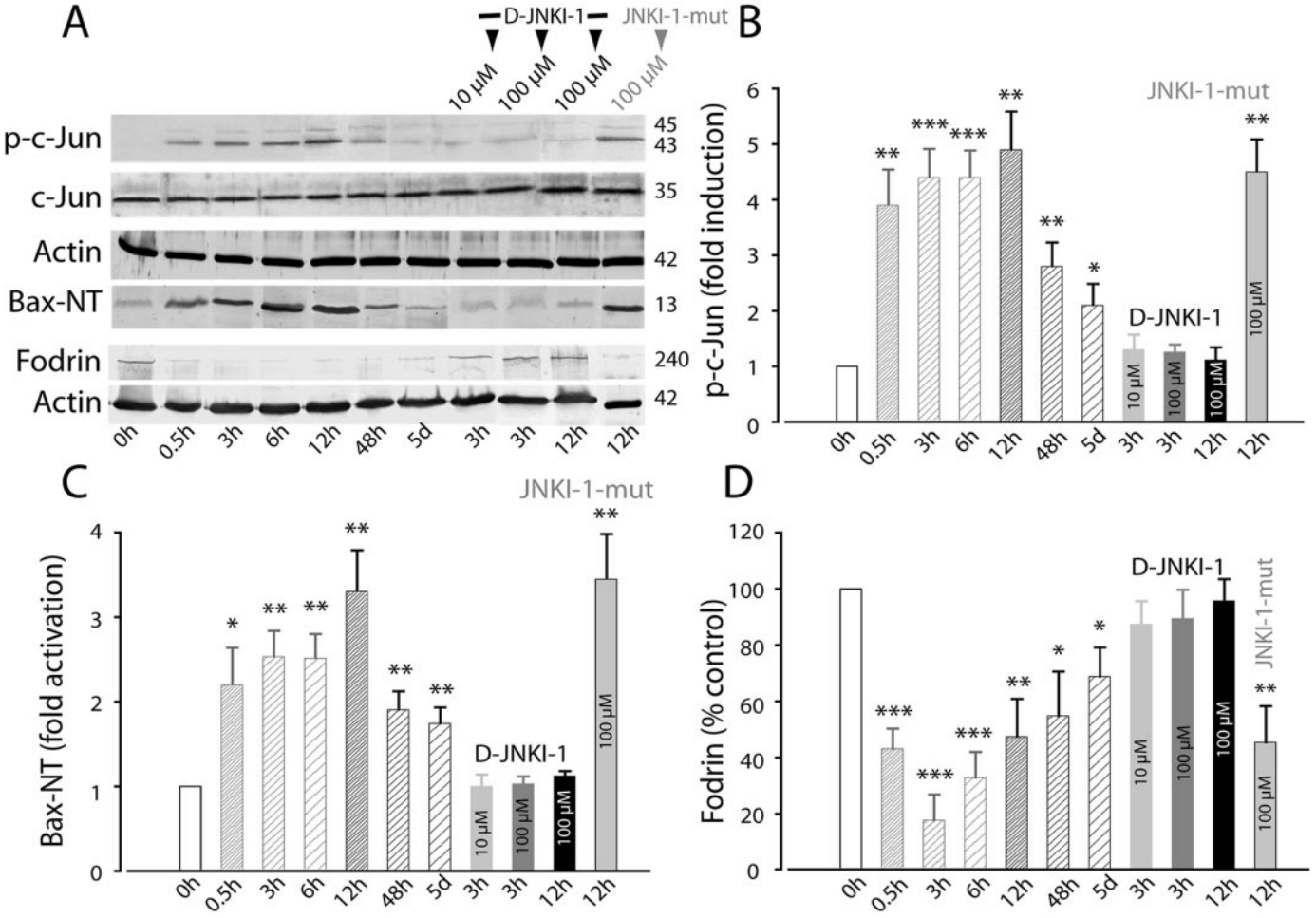

Fig. 3. JNK activation of c-Jun mediates the mitochondrial cell death pathway. A, representative Western blots using antibodies to phosphorylated c-Jun (p-c-Jun, Ser73) and the inactivated form of c-Jun, N-terminal of Bax that recognizes only the activated form of Bax or 240/280 kDa α-fodrin (all spectin) or β-actin in control non–sound-exposed, sound-exposed, sound-exposed treated with D-JNKI-1 peptide, and sound-exposed treated with JNKI-1mut (inactive form). B, histograms represent p-c-Jun/c-Jun ratios in sound-exposed cochleae expressed relative to the same ratio in non–sound-exposed cochleae (i.e., 0-h specimens). C, histograms represent the increase in Bax-NT after sound exposure. Data expressed as activation with respect to non–sound-exposed ears (i.e., 0-h specimens). D, histograms represent the levels of α-fodrin after sound exposure relative to control, non–sound-exposed ears (100%). In all cases, 10 or 100 μM concentration of D-JNKI-1 prevented phosphorylation of c-jun, activation of Bax and the cleavage of fodrin 3 and 12 h after sound exposure, whereas the inactive form of the peptide inhibitor (JNKI-1mut) had no effect ($p > 0.05$). All values are presented as means ± S.D. for all experiments ($n = 4$ per time point). ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$.

ligament clearly labeled for the presence of FITC-labeled D-JNKI-1, whereas the stria vascularis did not (Fig. 5, A–E).

Time-dependent fluorescence measurements revealed that the intensity of the FITC signal remained stable in all of the cochlear structures throughout the cochlear duct 6 h, 3 days, and 7 days after RWM delivery (Fig. 5, B, C, and H). A slight but not significant decrease in fluorescence occurred (i.e., 17%, Fig. 5, D and H) in all of the cochlear turns at 14 days, and there was a limited amount of fluorescence in the tissue of the cochlear basal turn at 21 days after the initial delivery (Fig. 5, E and H). Fluorescence from FITC-labeled D-JNKI-1 peptide was no longer observed in any of the cochlear turns at 28 days after administration (Fig. 5H).

Efficiency of RWM Delivery of D-JNKI-1 Peptide

Control Experiments. Control experiments were designed to demonstrate the lack of nonspecific effects that were due to surgery, minipump implantation, or the introduction into the cochlea of TAT vector or a peptide. In our studies, the implantation of a minipump containing D-JNKI-1, TAT-empty, or JNKI-1-mut induced a slight high-frequency hearing loss (5–10 dB) on the first day that had

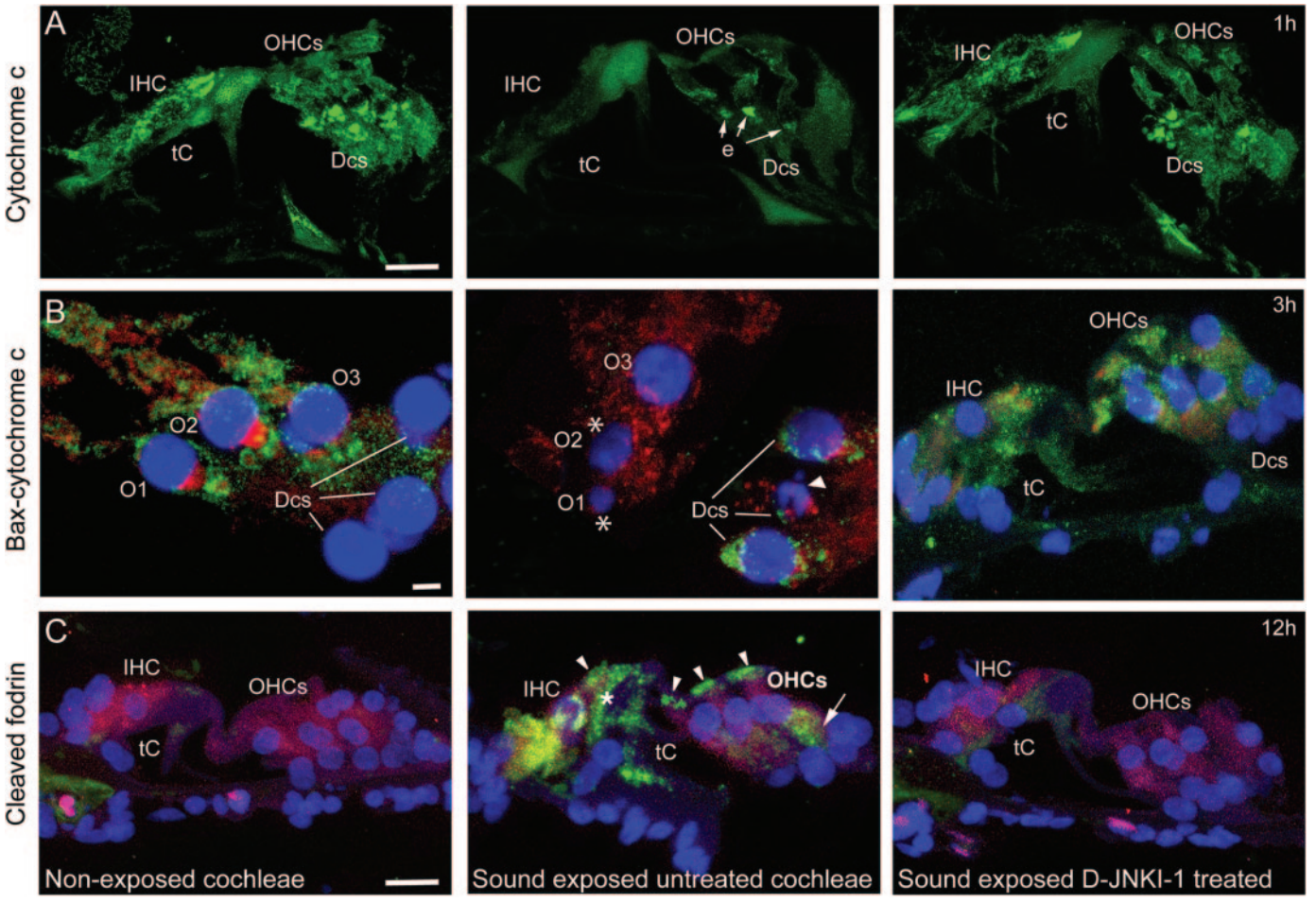

Fig. 4. Release of cytochrome *c*, translocation of Bax, and cleavage of α-fodrin in damaged sensory hair cells. A, confocal images of transverse cryostat sections of the organ of Corti labeled with antibody against cytochrome *c*. Left image shows a normal pattern of intense punctate staining for cytochrome *c* in both the IHC and the OHCs in nonexposed cochleae. In contrast, the middle image shows a diffuse pale pattern of immunostaining for cytochrome *c* in both the IHCs and the OHCs at 1 h after exposure, indicating a redistribution of cytochrome *c* from the mitochondria to the cytoplasm. Note that there is still a normal pattern of immunostaining for cytochrome *c* for the medial efferents that connect to the OHCs (white arrows and e). The right image is a sound-exposed cochlea that was treated with 100 μM concentration of D-JNKI-1 peptide via the RWM. Note the normal pattern of intense punctate staining for cytochrome *c* in both the IHC and the OHCs as in the nonexposed control cochleae. B, triple labeling of nonexposed, sound-exposed untreated, and sound-exposed D-JNKI-1 treated cochleae at 3 h after sound exposure. Cochlear cells were double-labeled for Bax (red) and cytochrome *c* (green) and counter-stained with Hoechst 33342 dye to label chromatin (blue). The left higher-magnification image shows a diffuse pattern of Bax immunostaining and intense punctate staining for cytochrome *c* in both the OHCs (O_1, O_2, and O_3) and Deiter cells. There is a lack of colocalization of cytochrome *c* and Bax immunolabeling with a uniform pattern of DNA staining in all of the hair cell and support cell nuclei. In contrast, the middle higher magnification image shows intense and punctate pattern of immunostaining for Bax and the weak pale pattern of immunostaining for cytochrome *c* in the OHCs. The DNA staining revealed condensed, shrunken, and pyknotic nuclei in two OHCs (O1 and O2, asterisks) and one Deiter cell (white arrowhead). Note that the damaged Deiter cell (white arrowhead) shows almost no cytochrome *c* staining, whereas the intact Deiter cells on either side display a punctate staining for this respiratory chain enzyme. The D-JNKI-1 treatment prevented the translocation of Bax in both the hair cells (IHCs, OHCs) and the support cells. C, triple labeling of nonexposed, sound-exposed untreated, and sound-exposed D-JNKI-1-treated cochleae at 12 h after sound exposure. Cochlear cells were double-labeled for α-fodrin (green) and anti-calbindin antibody (red) and were counterstained with Hoechst 33342 dye to label chromatin (blue). Compared with nonexposed cochlea (left image), sound-exposed untreated cochlea displays a robust immunostaining for the cleaved form of α-fodrin in the cuticular plates (arrowheads) and the cytoplasm of both the OHCs and the IHC and in the pillar cells (asterisk). The D-JNKI-1 treatment prevented caspase-induced cleavage of fodrin (right image). Dcs, Deiter cells; e, efferent fibers. Scale bar in A, B, and C = 10 μm, except for the left and middle images in B, where the scale bar = 2 μm.

completely recovered within 2 days after implantation (data not shown). To evaluate the functional consequences of drug delivery, treated right and untreated control left ears were exposed to sound trauma (6 kHz, 130 dB SPL, 15 min) via an earphone positioned 10 cm in front of the animal's head. Hearing losses were not significantly different between sound-exposed cochleae treated with TAT-empty or JNKI-1-mut and the untreated control left ears (Fig. 6A). Consequently, the protective effects of D-JNKI-1 were therefore compared with the untreated control left ear in the same animals.

Protective Effects against Sound Trauma

Functional Evaluation. We investigated the ability of D-JNKI-1 to protect hair cells against sound exposure when concentrations of increasing dosage levels of D-JNKI-1 (0.01–100 μM, six animals per dose) were applied onto the round window membrane. Sound-exposed untreated left cochleae served as controls for the effectiveness of sound-induced hair cell and hearing loss. In these cochleae, the impairment reached a maximum of 35 to 40 dB in the higher frequencies (8–16 kHz) of the CAP audiogram (Fig. 6A) when measured 15 days after sound exposure. This represents a permanent hearing loss [i.e., permanent threshold shift (PTS)]. When the round window membranes of the sound trauma-exposed cochleae were infused with 100 μM concentration of D-JNKI-1 in AP, there was still an immediate and significant elevation in hearing threshold (approximately 65 dB in the 8–16 kHz range) due to the initial effect of the sound trauma. However, this was followed by a clear improvement and almost complete recovery of the CAP thresholds when they were measured at 15 days after the initial sound exposure ($p < 0.05$ at 4–12 kHz, Fig. 6A) (i.e., this was only a temporary threshold shift).

Statistical analysis was performed on the data obtained at 8 kHz 15 days after sound trauma because this was the

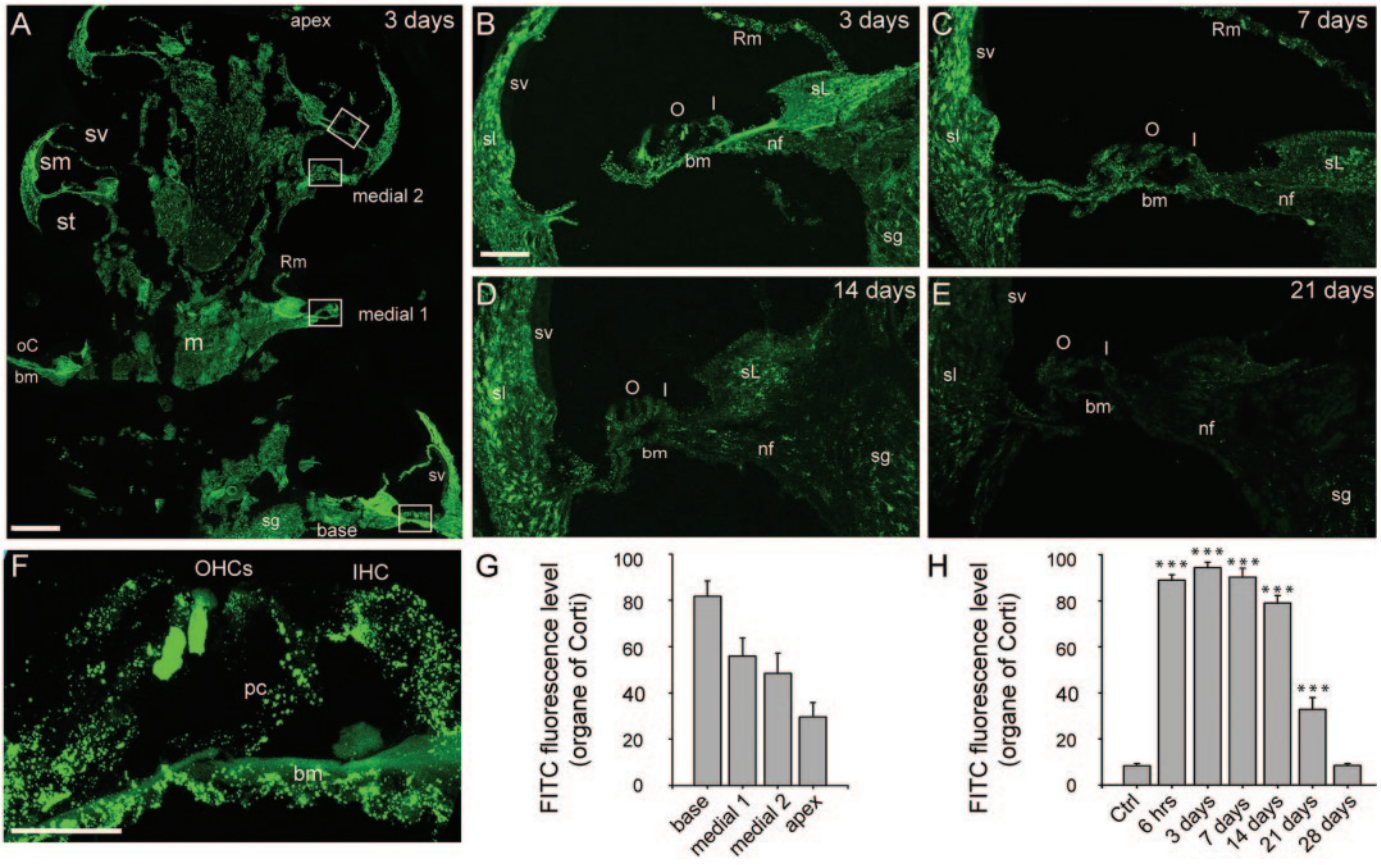

Fig. 5. FITC-conjugated D-JNKI-1 delivered onto the round window diffuses into the cochlea. A to F, FITC-conjugated D-JNKI-1 peptide (green) at a concentration of 100 μM in AP irrigated into the RWM niche for 30 min. A, green fluorescent staining is seen in the scala tympani (st), scala vestibuli (sv), and scala media (sm) of all cochlear turns 3 days after delivery. The FITC label shows a base-to-apex gradient of distribution. B to E, the intensity of the FITC signal remained intense and stable in the basilar membrane (bm), Reissner's membrane (Rm), the spiral limbs (sL), spiral ligament (sl), OHCs (O), IHCs (I), peripheral branches of the cochlear nerve fibers (nf), and spiral ganglion (sg) but not the cells of the stria vascularis (sv) at 3 days (in B) and 7 days (in C). In comparison, there was a slight decrease of FITC D-JNKI-1 labeling in the cochlear turns at 14 days after delivery (in D) and a further decrease was observed at 21 days after application (in E). F, higher magnification of organ of Corti at 3 days after delivery showing FITC-labeled D-JNKI-1 clearly visible in the OHCs and to a lesser extent within the IHC. G and H, quantitative analysis of FITC fluorescence levels in the organ of Corti of all four cochlear turns from guinea pig cochleae removed at 3 days (in G) and in the basal turn of organ of Corti specimens at 6 h and 3, 7, 14, 21, and 28 days after RWM application of FITC-conjugated D-JNKI-1 peptide (in H). FITC fluorescence levels measurements were performed using a histogram function in a 20 $\times$ 10-pixel box focused on organs of Corti of all turns that were identified by light microscopy images with projections of fluorescence microscopy. Intensity of fluorescence is expressed as the mean of pixel intensity (mean $\pm$ S.D.). G, there is a base-to-apex gradient pattern of distribution of FITC-labeled D-JNKI-1 3 days after administration. H, FITC fluorescence persists up to 14 days after application of the FITC-conjugated D-JNKI-1 peptide. These fluorescence levels drastically decrease at 21 days, and no difference was observed between the control untreated cochleae and the FITC-conjugated D-JNKI-1-RWM cochleae at 28 days. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$. oC, organ of Corti; m, modiolus; pc, pillar cells. Scale bars: A, 170 μm; B–E, 60 μm; F, 30 μm.

frequency at which the maximal PTS was seen in the sound-exposed untreated cochleae. Significant improvement was seen in CAP thresholds for all concentrations of D-JNKI-1 greater than 1 μM (Fig. 6E), and there was a complete recovery to pre-exposure hearing thresholds in response to treatment with 33 μM D-JNKI-1 (Fig. 6E). The effective concentrations of D-JNKI-1 required to prevent 50% of the permanent threshold shift (EC_{50}) caused by exposure to the sound trauma was 2.05 μM for round window delivery (Fig. 6E).

Morphological Evaluation. SEM observation of the surface morphology of each organ of Corti was performed at the end of the physiological assessment of hearing thresholds (i.e., 15 days after sound trauma). Counting all of the hair cells present over the entire length of the cochlear duct allowed us to construct cochleograms for the sound-exposed, contralateral untreated cochleae (Fig. 6B) and sound-exposed cochleae treated with 100 μM D-JNKI-1 in artificial perilymph (Fig. 6C). In the contralateral cochleae, more than 80% of the hair cells were missing from the area representing the site of maximal damage 14 to 16 mm from the apex (Fig. 6D). Application of 100 μM D-JNKI-1 resulted in protection of the hair cells from the effects of the sound trauma – there was no PTS in this group. Less than 15% of the hair cells were missing from the area of maximum sound-induced damage (Fig. 6D), which is consistent with the physiological data showing the development of almost no permanent loss of hearing.

Therapeutic Window for Effective D-JNKI-1 Treatment. Having demonstrated the efficacy of D-JNKI-1 as an otoprotective treatment applied during the sound trauma, we evaluated its ability to rescue cochlear function from a PTS when applied after the initial exposure to the sound trauma. D-JNKI-1 was applied at a concentration of 100 μM in artificial perilymph onto an intact RWM using an osmotic minipump over 7 days. Compared with the 100% protective effect of D-JNKI-1 applied 30 min before sound trauma, delaying the onset of the treatment until 30 min after the trauma led to an 84% protection of hearing threshold (Fig. 6F). This protective effect against sound trauma-induced hearing loss continuously diminished as the time interval between the initial exposure to the damaging level of sound and the onset of treatment increased (Fig. 6F). Calculation of the percentage of protection shows 80, 58, 41, 17, and 6% protection of hearing, respectively, with D-JNKI-1 RWM osmotic minipump therapy started 1, 4, 6, 12, and 24 h after exposure.

We also tested the ability of D-JNKI-1 to rescue the cochlea after sound exposure when it was administered onto the RWM in an HA gel. D-JNKI-1 delivered via this route enhanced functional recovery in a time-dependent manner (Fig. 6F). Furthermore, there was no significant difference in the protective effect on hearing between the D-JNKI-1 delivered via osmotic minipump or via an HA gel to the RWM (Fig. 6F).

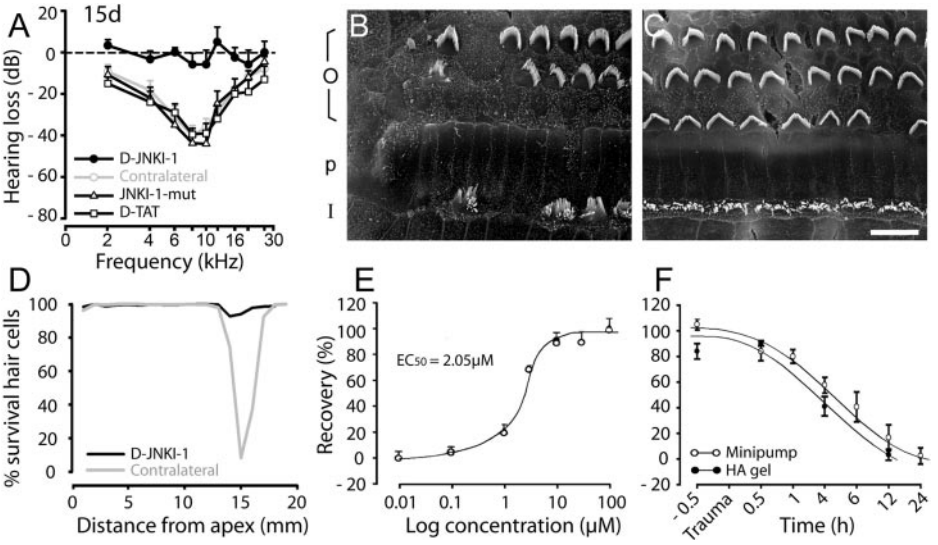

Fig. 6. D-JNKI-1 protects the cochlea against sound-induced hair cell loss and elevation of auditory threshold. A, hearing thresholds measured at 15 days after exposure from contralateral untreated, sound-exposed, left cochleae (◍), and the sound-exposed right cochleae that were infused with D-JNKI-1 (●), JNKI-1-mut (△), or D-TAT (□) onto the RWM. Contralateral untreated cochleae had a permanent hearing loss of 40 dB SPL (◍). Hearing loss was not significantly different in sound-exposed cochleae treated with D-TAT or JNKI-1-mut. In contrast, a significant improvement in the recovery of hearing threshold from an initial temporary threshold shift was seen in the D-JNKI-1-treated cochleae (●). B and C, scanning electron micrographs of the area of greatest damage in untreated and D-JNKI-1 treated cochleae from the same animal. Untreated cochlea shows severe damage to the IHCs (I) and to the first row of OHCs (O) with a gradation of damage extending to the second and the third rows of OHCs (in B). Application of D-JNKI-1 onto the RWM effectively prevented nearly all sound trauma-induced hair cell loss (in C). D, cochleograms representing the mean survival of hair cells as a function of the distance from the apex (in millimeters) in contralateral untreated cochleae (gray line) and in D-JNKI-1 treated cochleae (black line). In the sound-exposed untreated cochleae, more than 80% of the hair cells were missing in the maximally damaged area of the cochlea (14–16 mm from the apex). In contrast, less than 15% of the hair cells were lost in D-JNKI-1 treated cochleae. E, dose-response curve of D-JNKI-1 efficacy at day 15 after exposure. Hearing thresholds in response to an 8-kHz pure tone stimulus were expressed as the percentage of recovery. Dose-response data were fitted to a curve using a nonlinear least-square logistic equation, and the Boltzmann equation was used for fitting the sigmoid curves. The EC_{50} value was calculated as 2.05 μM for RWM delivery of D-JNKI-1. F, time-dependent effect of RWM administration of D-JNKI-1 via an osmotic minipump (○) or via HA gel (●) on functional recovery of hearing thresholds. D-JNKI-1 treatments were commenced 0.5 hours before or 0.5, 1, 4, 6, 12, or 24 h after sound exposure. Note the efficacy of D-JNKI-1 when treatment was started within 6 h after the initial trauma. There is no significant difference in effectiveness with method of delivery (i.e., osmotic minipump/RWM versus HA gel/RWM). Scale bars: B and C, 10 μm.

Discussion

Identification of Sensory Hair Cell Death. Several studies have implicated apoptosis in the death of hair cells after sound trauma (Hu et al., 2000; Pirvola et al., 2000; Wang et al., 2002; Cheng et al., 2005). However, the time course and proportion of apoptosis versus necrosis is only starting to be defined (Yang et al., 2004; Hu et al., 2006). Morphological changes in cell nuclei are the primary criteria for the differentiation of apoptosis and necrosis (Kerr et al., 1972). Condensed and fragmented nuclear DNA characterizes apoptosis, whereas swollen nuclei are characteristic of necrosis. Based on these criteria, we report a significant increase in apoptotic cell nuclei 1 h after sound trauma, which concurs with the observations in several recent publications studying the biological effects of sound and impulse sound trauma (Yang et al., 2004; Hu et al., 2006). After 2 days, the number of apoptotic cells dramatically decreased, probably due to rapid degeneration of cells in the damaged area, whereas necrotic nuclei reached a maximum of 8% of the total damaged cells. Ultrastructural investigations revealed typical features of apoptosis and of autolysis. Signs of necrosis were also occasionally seen in the traumatized area of the cochlea. It is interesting that some individual hair cells shared both necrotic and apoptotic features, which may be explained by the occurrence of a secondary necrotic process as a result of a metabolism deficiency, resulting in an inability to maintain the apoptotic process in the damaged cells (Leist and Jaattela, 2001). Although probably several mechanisms of cell death occurred (Leist and Jaattela, 2001; Lefebvre et al., 2002; Hetz et al., 2005; Krantic et al., 2005), our results suggest that apoptosis is the predominant mode of hair cell death after sound trauma. This mode of cell death may be linked to a defense mechanism that preserves the integrity of the cellular barriers, which avoid potassium-rich endolymph entering the sensory epithelia (Li et al., 1995). Finally, these degenerative mechanisms might have different time courses, which would explain the extended therapeutic window seen in which D-JNKI-1 can be administered up to 12 h after trauma and still protect against sound trauma-induced hearing loss.

JNK-Mediated Mitochondrial Cell Death Pathway. The c-Jun NH_2-terminal kinases represent one subgroup of mitogen-activated protein kinases that are primarily activated by cytokines and by exposure to environmental stress (Derijard et al., 1994; Kyriakis et al., 1994). There is increasing evidence that a major role for this JNK subgroup of kinases is as mediators of apoptosis (Ip and Davis, 1998; Mielke and Herdegen, 2000). A major target of the JNK signaling pathway is the activator protein-1 transcription factor, activation of which is mediated in part by phosphorylation of c-Jun and related molecules.

In this study, sound trauma induced a significant increase in phosphorylation of the nuclear transcription factor c-Jun, the activation and translocation of Bax into the mitochondria, the release of cytochrome *c* from the mitochondria into the cytoplasm of a damaged cell, and the cleavage of fodrin by activated effector caspases. It is interesting that there was still a normal pattern of immunostaining for cytochrome *c* for the medial efferent that connects to the OHCs, which concurs with previous ultrastructural studies reporting normal medial efferent appearance even after severe sound-induced damage in the OHC (Spoendlin, 1970; Fig. 2I).

Blocking the JNK/stress-activated protein kinase-mediated mitochondrial cell death pathway with D-JNKI-1 prevented both hair cell death and the permanent loss of hearing induced by sound trauma. This supports previous results (Maundrell et al., 1997; Ghatan et al., 2000), which demonstrated that MAPK kinase regulates the activation and translocation of Bax to mitochondria. However, we cannot exclude that other proapoptotic proteins of the Bcl-2 family such as Bim may be implicated in the JNK-mediated cochlear cell death as shown in other systems (Putcha et al., 2003). We did not study the activation of Bim by JNKs in the stressed cochlea, and thus, further experiments are necessary to clarify this point.

The cleavage of fodrin by effector caspases is consistent with a previous study reporting that sound-induced apoptotic sensory hair cell death is associated with the activation of procaspase-3 (Hu et al., 2002b) and cleavage of F-actin in affected outer hair cells (Hu et al., 2002a). The results of this study suggest that JNK activates the intrinsic mitochondrial cell death pathway and that this pathway is one of the major intracellular cascades by which hair cells respond to noise-induced damage.

Efficacy of RWM Delivery of D-JNKI-1. D-JNKI-1 inhibitory peptide contains a 10-amino acid human immunodeficiency virus-TAT transporter sequence to facilitate its entry into cells (Vives et al., 1997). FITC-conjugated D-JNKI-1 infused onto the RWM niche diffused rapidly through the RWM into the scala tympani. The fluorescent signal showed a gradient from base to apex in the scala tympani, media, and vestibuli. These results are consistent with those of Salt and Ma (2001), who used a trimethylphenylammonium-sensitive microelectrode to measure the concentration of this ion when applied onto the RWM. Most of the cellular subtypes within the organ of Corti, including the hair cells, exhibited fluorescence, indicating incorporation of FITC-labeled D-JNKI-1. The timed histological specimen study showed an initial cellular uptake of D-JNKI-1 as early as 30 min after its application onto the RWM and that this drug remained within cochlear cells for up to 3 weeks.

The fact that FITC-labeled D-JNKI-1 was still detectable in the cochlea at 3 weeks after only a single application offers interesting perspectives for a clinical application, because delivery of pharmacological agents onto the RWM in humans is already occurring in clinical practice (Seidman and Van De Water, 2003). Delivery of D-JNKI-1 onto the RWM preserved more than 80% of the hair cells and prevented the occurrence of permanent deafness. Using RWM delivery, 2.05 μM D-JNKI-1 was required to prevent 50% of the permanent hearing loss caused by our sound trauma. Compared with intracochlear infusion, which has an EC_{50} of 2.31 μM (Wang et al., 2003), the RWM route for the delivery of D-JNKI-1 therapy is as effective as direct intracochlear infusion. This observation is of great clinical importance and highly relevant to the use of this drug in a clinical setting.

Most of the studies investigating JNK inhibitors have examined their effectiveness when treatment commences before sound trauma (Pirvola et al., 2000; Ylikoski et al., 2002; Wang et al., 2003). Here, we report that D-JNKI-1 can not only prevent but also rescue the cochlea from the deleterious effects of sound trauma within a therapeutic window of 12 h

after trauma. In addition to the clinical relevance of a 12-h window of opportunity for the rescue of hearing, there are significant implications regarding the mechanism of cell death induced by sound trauma. This therapeutic window is consistent with the fact that JNK-mediated activation of the mitochondrial cell death pathway can occur between 30 min and 12 h after sound exposure. Analysis of our data revealed that during this time period, only 22% of the hair cells were missing, and 40% of the hair cells were apoptotic in the area of sound damage. One possible explanation is that when the apoptotic process is engaged, blockage of the JNK-activated mitochondrial signal pathway is no longer effective even if hair cells are still present, because JNK acts upstream in the apoptotic cascade (Jin et al., 2006). Further studies need to be undertaken to verify whether targeting a "downstream" event in the apoptotic cascade, such as activation of caspases, may prolong the window for initiation of therapy while retaining similar efficacy. If true, this might explain the 3-day therapeutic window reported by Yamashita et al. (2005) when using a combination of antioxidants (salicylate and trolox intraperitoneally) against sound trauma.

This study shows that the activation of MAPK/JNK signal cascade by sound trauma regulates the intrinsic cell death pathway involving the mitochondria. A single dose of the peptide inhibitor D-JNKI-1 onto the RWM diffused into and remained within the auditory hair cells for as long as 3 weeks, with the latter method of application being as potent as direct intracochlear perfusion of this peptide inhibitor of JNK in preventing sound-induced hair cell loss and deafness. The ability of D-JNKI-1 to diffuse through the RWM into auditory hair cells and neurons, the extended period of retention of this inhibitory compound within these neurosensory cells, and the finding of a 12-h post-trauma window for effective treatment provide an interesting clinical perspective and an opportunity to develop a realistic and effective therapy with which to treat cochlear injury. As has been demonstrated in this study, RWM delivery is the preferred method of drug delivery because it avoids any unwanted side effects that could be associated with systemic administration and avoids any local risk to the cochlea if the cochlea had to be opened to be infused. The HA gel method of drug delivery at the RWM is particularly attractive because HA gels have been demonstrated to be nontoxic, and it can be achieved using current surgical approaches without implantation of a pump system.

Acknowledgments

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