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Mechanism Underlying the Protective Effect of Tempol and N^{ω}-Nitro-L-arginine Methyl Ester on Acoustic Injury: Possible Involvement of c-Jun N-Terminal Kinase Pathway and Connexin26 in the Cochlear Spiral Ligament

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Abstract. There is evidence that reactive oxygen species (ROS) are formed in the cochlea during acoustic injury. However, very little is known about the involvement of ROS signals in the spiral ligament (SL) during such injury. The purpose of this study was to determine the effect of the multifunctional antioxidant tempol and the nitric oxide synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) on acoustic injury and the c-Jun N-terminal kinase (JNK) pathway in the SL. Exposure of adult mice to noise (8-kHz octave band, 110-dB SPL for 1 h) produced permanent hearing loss. Noise exposure increased not only the formation of a protein modified by 4-hydroxynonenal and formation of nitrotyrosine, but also the level of phospho-JNK in the SL. Pretreatment with tempol or L-NAME was effective in protecting the noise-exposed animals from hearing loss, as well as in abolishing the noise-induced activation of the JNK signaling pathway. Interestingly, noise exposure caused a dramatic decrease in connexin26 level in the SL. This decrease was prevented by tempol or L-NAME. Taken together, our data suggest that noise-induced hearing loss is due at least in part to ROS / nitric oxide–mediated activation of the JNK pathway and down-regulation of connexin26 in the SL of mice.

Keywords: c-Jun N-terminal kinase (JNK), cochlear spiral ligament, connexin, noise-induced hearing loss, nitric oxide (NO)

Introduction

The cochlear lateral wall is known to play important roles in maintaining the endocochlear potential, ion transport, and regulation of endolymph balance. Previous reports demonstrated that acoustic overstimulation produces, in addition to the shift in auditory brainstem response (ABR) threshold due to an altered endocochlear potential, pathologic changes in the cochlear lateral wall including microcirculation dysfunction, strial swelling, and degeneration of intermediate and marginal cells (1). These findings suggest that acoustic injury is attributed at least in part to the abnormal endocochlear potential resulting from dysfunction of the stria vascularis and spiral ligament in the cochlear lateral wall. However, the mechanisms underlying lateral wall damage induced by acoustic overstimulation is not well understood.

Accumulating evidence suggests that reactive oxygen species (ROS) / nitric oxide (NO) are generated in the cochlea and involved in the pathogenesis of acoustic injury in the cochlea. Ohlemiller et al. (2, 3) demonstrated that acoustic overstimulation increases the generation of free radicals in the cochlea and that the superoxide dismutase knockout mouse has high susceptibility to acoustic injury. In addition, acoustic overstimulation enhances the expression of inducible NO synthase (NOS3) in the stria vascularis and hair cells of the cochlea in mice and guinea pigs (4). Furthermore, the ROS scavenger tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl) or the NOS inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) decreases the ABR threshold shift caused by acoustic overstimulation (5, 6). c-Jun N-termi-

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nal kinase (JNK) / stress–activated protein kinase is an important mediator of apoptosis in different model systems (7 – 9). The JNK pathway is activated in response to free radicals generated by UV radiation, exposure to inflammatory cytokines, and direct application of H_2O_2 (10). Upstream activators of JNK have been identified by investigations conducted over the past few decades. JNKs are activated through phosphorylation of their threonine 183 and tyrosine 185 residues by stress-activated protein kinase 1 (SEK-1), which is also known as extracellular-signal regulated kinase kinase or mitogen-activated protein kinase kinase (MKK) 4. As the downstream, JNKs activate the inducible transcription factor c-Jun and ATF-2, which are involved in multiple cellular functions such as survival, differentiation, and apoptosis (11). A recent study demonstrated that the activation of c-Jun by JNK is a central event in the JNK-mediated apoptosis of oxidative stress–damaged auditory hair cells following exposure of the cells to either acoustic injury or a toxic level of an aminoglycoside antibiotic, as well as in the apoptosis of auditory neurons as a consequence of a loss of the trophic support provided by the auditory hair cells (12).

To date, however, very little has been elucidated regarding ROS/NO–induced activation of the JNK pathway in the lateral wall following acoustic overstimulation. The objective of the present study was to investigate whether ROS/NO–induced activation of the JNK pathway is involved in the dysfunction of the lateral wall following acoustic overstimulation. To this end, we determined the preventive effect of tempol and L-NAME on intense noise–induced events such as oxidative stress and activation of the JNK pathway in the lateral wall as well as hearing loss in mice. In addition to the preventive effect of these drugs, there was a dramatic decrease in the level of connexin26, a major gap junction protein, in the lateral wall after intense noise exposure, which was abolished by tempol or L-NAME.

Materials and Methods

Materials

Tempol and L-NAME were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The rabbit polyclonal antibody against 4-hydroxynonenal (4-HNE) was supplied by Merck KGaA (Darmstadt, Germany). Antibodies against nitrotyrosine (mouse monoclonal) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, sc-25778, rabbit polyclonal) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Mouse monoclonal antibody against connexin26 was purchased from Zymed Laboratories, Inc. (South San Francisco, CA, USA). Rabbit polyclonal antibodies against JNK, phospho-JNK (p-JNK, Thr183/Thr185), phospho-SEK-1/MKK4 (p-SEK-1, Thr261), phospho-c-Jun (p-c-Jun, Ser63), and phospho-ATF-2 (p-ATF-2, Thr71) were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Rhodamine-phalloidin was from Invitrogen Co. (Eugene, OR, USA); diaminobenzidine / hydrogen peroxide solution (Histofine) from Nichirei Co. (Tokyo); and the streptavidin-biotin complex peroxidase kit, from Nacalai Tesque, Inc. (Kyoto). Polyvinylidene fluoride membranes (Immobilon-P) were obtained from Millipore (Bedford, MA, USA). Western Lightning Chemoluminescence Reagent Plus was purchased from Perkin-Elmer Life Science Products, Inc. (Boston, MA, USA). All other chemicals used were of the highest purity commercially available.

Animal and drug treatment

The protocol used here met the guidelines of The Japanese Pharmacological Society and was approved by the Committee for Ethical Use of Experimental Animals at Setsunan University. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to in vivo techniques. Adult male Std-ddY mice weighing 26 – 28 g, which we routinely used for neuroscience studies, were housed in metallic breeding cages in a room with a light–dark cycle of 12 h–12 h and a humidity of 55% at 23°C and given free access to food and water. To remove animals with natural auditory impairment, we measured their ABR before use and selected those animals with normal acoustic sense in the present study. Drugs were intraperitoneally injected into mice 30 min before noise exposure.

Intense noise exposure

Animals were exposed to 90, 100, 110, or 120 dB sound pressure levels (SPLs) of octave-band noise, centered at 8 kHz, for 1 h within a sound chamber. Each animal was placed in a cage (13). The sound chamber was fitted with a speaker (300HT; Fostex, Tokyo) driven by a noise generator (SF-06; Rion, Tokyo) and power amplifier (DAD-M100proHT; Flying Mole, Shizuoka). To ensure uniformity of the stimulus, we calibrated and measured the sound levels with a sound level meter (NL-26, Rion). The sound level meter was positioned at the level of the animal's head. As the control, naïve animals were placed in the same cage without noise.

ABR recording

For ABR measurement, stainless steel needle electrodes were placed at the vertex and ventro-lateral to the left and right ears. Electroencephalogram recording was performed with the extracellular amplifier Digital Bioamp system (BAL-1; Tucker-Davis Technologies, Ala-

chua, FL, USA), and waveform storing and stimulus control were performed by using Scope software of the Power Lab system (Power Lab 2/20; AD Instruments, Castle Hill, Australia). Sound stimuli were produced by a coupler type speaker (ES1spc; Bioresearch Center, Nagoya) inserted into the external auditory canal of a mouse. Tone burst stimuli, 0.1-ms rise/fall time (cosine gate) and 1-ms flat segment, were generated by using a real-time processor (RP2.1, Tucker-Davis Technologies), and the amplitudes were specified by a programmable attenuator (PA5, Tucker-Davis Technologies). Sound levels were calibrated with a sound level meter (TYPE 6224; ACO Co., Ltd., Tokyo). ABR waveforms were recorded for 12.8 ms at a sampling rate of 40,000 Hz using 50 – 5000 Hz by bandpass filter settings. Waveforms from 500 stimuli were averaged. For recording, animals were anesthetized (500 mg/kg chloral hydrate, i.p.). The thresholds of ABR were determined before noise exposure and immediately, 1, 2, 5, and 7 days afterward at 4, 12, and 20 kHz, by using a 5-dB SPL minimum step size down from the maximum amplitude. The hearing threshold was defined as the lowest stimulus intensity that produced a reliable wave III of the ABR. Because the constraining test tones were set to SPLs of less than 90 dB at 4, 12, and 20 kHz, respectively, the thresholds were recorded as 100 dB, respectively, for the calculation of the threshold shift value when there was no response due to profound hearing impairment.

Histological assessment

Mice were sacrificed by cervical dislocation, and their temporal bones were then removed. The round and oval windows and the apex of the cochlea were opened and then perfused with Bouin's solution [picric acid : 37% (vol/vol) formaldehyde : acetic acid = 15:5:1]. The tissues were subsequently kept at room temperature overnight. The post-fixed cochlea was embedded in paraffin, and then sections at 5-μm thickness were prepared by using a microtome. The sections were deparaffinized with xylene and then rehydrated by passage through ethanol at graded concentrations of 50% – 100% (vol/vol) and then immersion in water. Immunoreactivity was determined by the avidin–biotin-peroxidase method. For the immunostaining of nitrotyrosine and connexin26, the sections were washed with Tris-buffered saline (pH 7.5) containing 0.03% (wt/vol) Tween 20 (TBST) and then incubated with 0.03% (vol/vol) H_2O_2 in methanol for 5 min. After blocking with 10% (vol/vol) normal goat serum in TBST for 1 h at room temperature, the sections were sequentially incubated with mouse monoclonal antibody against nitrotyrosine (1:200) or connexin26 (1:200) at 4°C overnight, with biotinylated anti-mouse IgG antibody for 30 min at room temperature, and then with ABC solution for 1 h at room temperature. For the immunostaining of p-JNK, p-SEK-1, p-c-Jun, and p-ATF-2, the sections were first treated by the microwave irradiation and subsequently washed with TBST and then incubated in 0.03% (vol/vol) H_2O_2 in methanol for 5 min. After blocking with 10% (vol/vol) normal goat serum in TBST for 1 h at room temperature, the sections were sequentially incubated with rabbit polyclonal antibody against p-JNK (1:100), p-SEK-1 (1:100), p-c-Jun (1:100), or p-ATF-2 (1:250) at 4°C overnight, with biotinylated anti-rabbit IgG antibody for 30 min at room temperature, and then with ABC solution for 1 h at room temperature. For visualization, the peroxidase reaction was performed by using diaminobenzidine / hydrogen peroxide solution.

Histopathology of the hair cells was assessed by making surface preparations. On day 14 after noise exposure, mice were sacrificed by cervical dislocation, and the temporal bones were removed. The round and oval windows and the apex of the cochlea were opened and then perfused with 4% paraformaldehyde in 10 mM phosphate–buffered saline (PBS, pH 7.4). The tissues were kept in this fixative overnight at 4°C. The cochlea was then washed with 0.3% (wt/vol) Triton X-100 in PBS for 10 min. Thereafter, the organ of Corti in the basal turn was separated from the lateral wall and the modiolus and then stained with rhodamine-phalloidin (a stock solution of 200 U/mL methanol diluted 1:100 in PBS) on a slide glass.

Immunoblot analysis

Cochlear spiral ligaments were quickly removed and immersed in ice-cold homogenizing buffer consisting of 10 mM Tris-HCl buffer (pH 7.5), 0.32 M sucrose, 1 mM EDTA, 1 mM EGTA, 5 mM dithiothreitol (DTT), phosphatase inhibitors (10 mM sodium β-glycerophosphate and 1 mM sodium orthovanadate), and 1 μg/mL each of protease inhibitors [(p-amidinophenyl)methanesulfonyl fluoride, benzamidine, leupeptin, and antipain]; homogenized in 30 μL of the homogenizing buffer; and then immediately boiled for 10 min in a solution comprising 2% (wt/vol) SDS, 5% (vol/vol) 2-mercaptoethanol, 10% (vol/vol) glycerol, and 0.01% (wt/vol) bromophenol blue (14). The samples were stored at −80°C until used for immunoblot analysis. Protein concentrations were determined by use of the Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA, USA).

The immunoblot analysis was carried out as described previously (15). Briefly, an aliquot of sample was loaded onto a 10% (wt/vol) polyacrylamide gel for detection of JNK and p-JNK. Proteins were transferred to a polyvinylidene fluoride membrane and blocked with 5% (wt/vol) skim milk dissolved in washing buffer [Tris-buffered

saline containing 0.05% (wt/vol) Tween 20]. The membranes were incubated with the desired primary antibody for 2 h at room temperature, then washed with the above washing buffer for 3 cycles of 5 min each, and subsequently incubated with horseradish peroxidase–conjugated antibody against rabbit IgG for 1 h at room temperature. Proteins reactive with the antibody were detected with the aid of Western Lightning Chemoluminescence Reagent Plus and visualization by exposure to X-ray films.

Na^+, K^+-ATPase activity

Na^+, K^+-ATPase activity was determined by colormetrically measuring the amount of inorganic phosphate released from the substrate ATP (16). Cochlear lateral wall was quickly removed and homogenized in ice-cold HEPES buffer (pH 7.5). The homogenate (6 μg protein) was incubated 10 min at 37°C in reaction buffer consisting of 40 mM Tris-HCl buffer (pH 7.5), 150 mM KCl, and 1.2M NaCl in the absence or presence of 100 μM ouabain and then further incubated 30 min at 37°C in the reaction buffer containing 10 mM ATP and 30 mM $MgSO_4$. The reaction was terminated by the addition of 12% perchloric acid and 0.84% ammonium molybdate to measure released inorganic phosphate. The specific activity was calculated from the values obtained in the absence and presence of ouabain and expressed as μmol·min^{-1}·mg protein^{-1}.

Data analyses

The area under the curve was calculated by analyzing the densitometric data by the software Lane Analyzer ver. 3 (Rise Co., Ltd. & Atto Corp., Tokyo). Each result was expressed as the mean ± S.E.M., and the statistical significance of differences was determined by one-way ANOVA with the Bonferroni/Dunnett *post hoc* test or the Mann-Whitney *U*-test.

Results

ABR threshold shift and hair cell loss following exposure to noise at different SPLs

To determine hearing loss and hair cell damage following noise exposure, we measured the ABR immediately and on day 7 after a 1-h exposure to noise at 90-, 100-, 110-, or 120-dB SPLs (Fig. 1). Noise was effective in shifting the ABR threshold at the frequencies of 4, 12, and 20 kHz immediately after exposure (Fig. 1a, full line). Immediately post-exposure, the ABR threshold shifts were from 20 to 80 dB at all frequencies, which were progressively increased in an SPL-dependent manner. The ABR threshold shifts induced by noise at 110- and 120-dB SPL remained at more than 20 dB even on day 7 post-exposure. However, no significant ABR threshold shift was seen on day 7 post-exposure to noise at 90- and 100-dB SPL. These results suggest that noise at lower SPL (90 and 100 dB) and higher SPL (110 and 120 dB) produced a temporary and permanent threshold shift, respectively.

To determine hair cell death in the cochlea, we fixed inner ears on day 14 post–noise exposure and then stained them with rhodamine-phalloidin (Fig. 1b). In naïve animals, the basal turn in the organ of Corti had intact outer

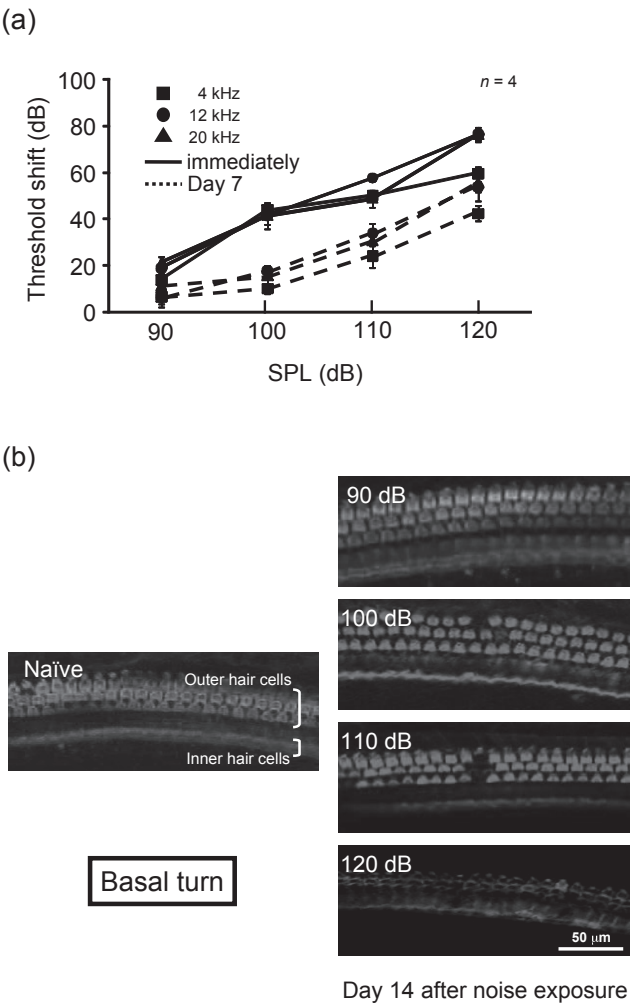

Fig. 1. Hearing loss and hair cell damage following noise exposure. Animals were exposed for 1 h to noise at the various SPLs indicated and then subjected to ABR threshold and histological assessments. a) Immediately (full lines) or on day 7 (dotted lines) after noise exposure at different SPLs, the ABR threshold was assessed at the frequencies of 4, 12, and 20 kHz. The graph denotes the average of threshold shift at each frequency. Values are the means ± S.E.M. from 4 independent experiments. b) On day 14 after noise exposure at various SPLs, animals were fixed for dissection of the organ of Corti, whose basal turn was histologically assessed by rhodamine-phalloidin staining. These experiments were carried out at least 3 times with similar results obtained under the same experimental conditions.

and inner hair cells labeled with phalloidin. Expectedly, marked loss of outer hair cells was seen in the cochlea of animals exposed to noise at 120-dB SPL. Further histological assessment in the lateral wall structures using hematoxylin-eosin staining and Hoechst staining under light microscopic observation revealed that no marked histological change was observed in any structures of the lateral wall at least until day 14 post-exposure under the experimental conditions used (data not shown).

Increased level of 4-HNE–protein adduct and nitrotyrosine in the cochlea following noise exposure

Earlier reports indicated that noise-induced hearing loss and that due to ototoxic drugs are elicited at least in part due to intensive oxidative stress (17, 18). To determine if intensive oxidative stress in the cochlea occurred following noise exposure, we performed immunoblot analysis of 4-HNE–protein adducts; 4-HNE is the major aldehydic product of lipid peroxidation and believed to be largely responsible for the cytopathological effects observed during oxidative stress (19, 20) in the cochlea following noise exposure at 110-dB SPL (Fig. 2). Numerous 4-HNE–protein adducts were found in the lateral wall and modiolus/organ of Corti of naïve animals. Of these proteins, a 50 – 60-kDa protein increased in both the lateral wall and modiolus/organ of Corti predominantly immediately post-exposure.

Nitrotyrosine is a product of tyrosine nitration mediated by reactive nitrogen species such as peroxynitrite anion and nitrogen dioxide (21). It is detected in a number of pathological conditions and is considered a marker of NO-dependent oxidative stress. To determine the expression of nitrotyrosine following noise exposure, we thus carried out immunostaing of nitrotyrosine in the cochlea at various times post-exposure to noise at 110-dB SPL (Fig. 3). Noise exposure produced an elevation of nitrotyrosine in the spiral ganglion cells as well as in the lateral wall including the stria vascularis (SV) and spiral ligament (SL) at 4 h afterward (Fig. 3a). Although noise exposure had the ability to induce swelling in supporting cells of the organ of Corti, unexpectedly, no marked elevation of nitrotyrosine immunoreactivity was seen in the organ of Corti of noise-exposed animals. Time-course experiments revealed a marked increase in the level of immunoreactive nitrotyrosine in the lateral wall from 4 to 12 h post–noise exposure (Fig. 3b). However, the immunoreactivity retuned to the level in naïve animals at least by 24 h post-exposure.

Effect of tempol and L-NAME on ABR threshold shift and hair cell loss following noise exposure

To assess the effect of tempol and L-NAME on the ABR threshold shift (Fig. 4a) and hair cell loss (Fig. 4b)

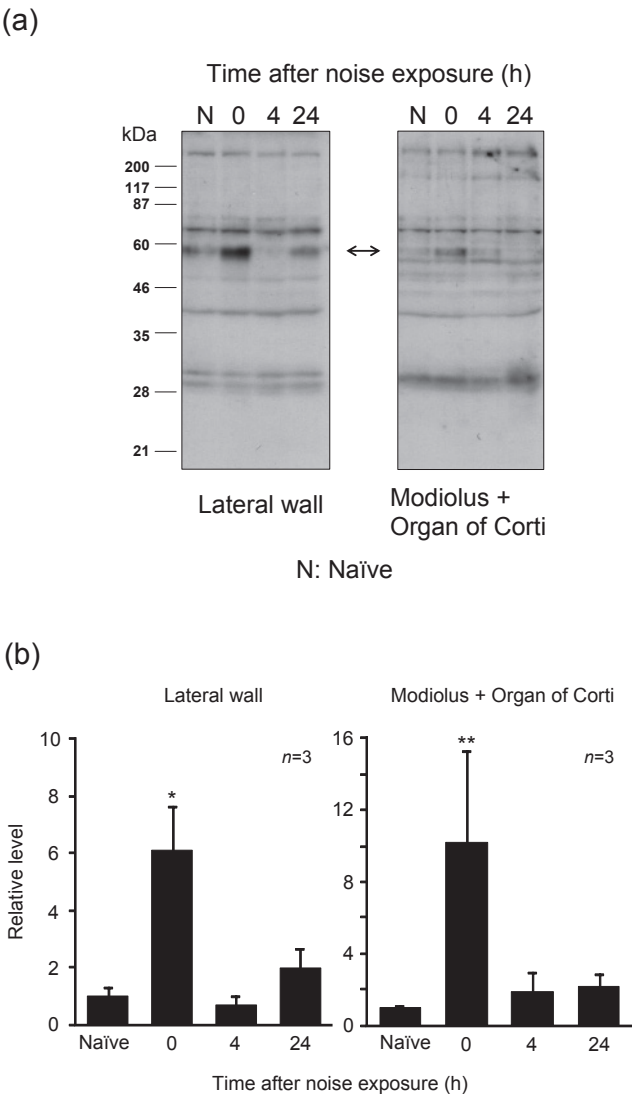

Fig. 2. Increased level of the 4-HNE–protein adducts in the cochlea following noise exposure. Animals were exposed to noise at 110-dB SPL for 1 h and then decapitated at various time points after noise exposure for immunoblot analysis of 4-HNE–protein adducts in homogenates prepared from the "lateral wall" and "modiolus + organ of Corti". a) Typical data of the immunoblot analysis show an increase in the level of the 4-HNE–protein adduct indicated by the double-headed arrow after noise exposure. b) Graphs denote quantitative data on the protein indicated by the double-headed arrow. Values are the means ± S.E.M. from 3 independent experiments. $*P < 0.05$, $**P < 0.01$, significantly different from each control value obtained for naïve animals.

following noise exposure, we treated mice with either tempol or L-NAME at different doses at 30 min before exposure to noise at 110-dB SPL. Higher doses of tempol (3 mg/kg or more) or L-NAME (0.1 mg/kg or more) were capable of attenuating the threshold shift induced by noise exposure. Surprisingly, the higher doses of either drug completely prevented ABR threshold shifting at all

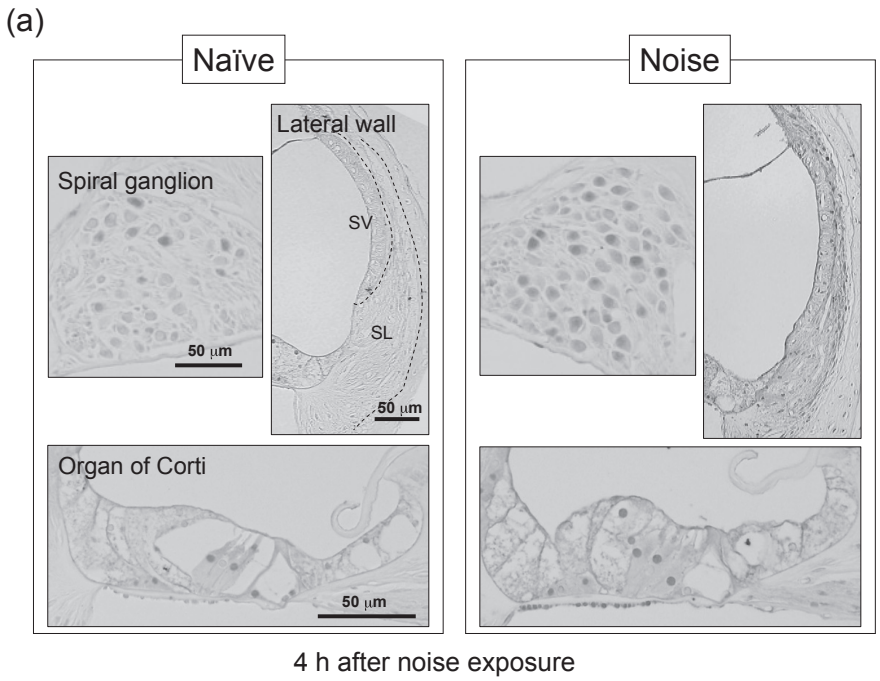

Fig. 3. Increased level of nitrotyrosine in the cochlea following noise exposure. Animals were exposed to noise at 110-dB SPL for 1 h and then fixed at the various time points indicated after noise exposure for immunostaining of nitrotyrosine in slices prepared from the cochlea of naïve or noise-exposed animals. a) Immunostaining of nitrotyrosine in the spiral ganglion, lateral wall (SV, stria vascularis; SL, spiral ligament), and organ of Corti of naïve and noise-exposed animals. b) Time course of nitrotyrosine immunoreactivity in the lateral wall after noise exposure. These experiments were carried out at least 3 times, with similar results obtained under the same experimental conditions.

frequencies tested on day 7 post–noise exposure. However, the lowest dose of tempol (0.3 mg/kg) or L-NAME (0.01 mg/kg) used was ineffective in decreasing the shift at any frequency tested from immediately to day 7 post-exposure.

On day 14 post–noise exposure, expectedly, the noise-induced outer hair cells in the basal turn of the organ of Corti were dramatically protected against noise-induced loss by pretreatment with tempol (30 mg/kg) or L-NAME (1 mg/kg, Fig. 4b).

Effect of tempol and L-NAME on activation of the JNK pathway in the spiral ligament following noise exposure

To assess whether noise exposure had activated JNK in the lateral wall, we performed immunostaining (Fig. 5a) and immunoblot (Fig. 5b) analysis for JNK and p-JNK at various times after noise exposure at 110-dB SPL. Immunostaining revealed a marked elevation of p-JNK immunoreactivity in the spiral ligament and stria vascularis, with a peak at 2 h post–noise exposure. Like the immunostaining, immunoblot analysis revealed that the level of p-JNK significantly increased in the lateral

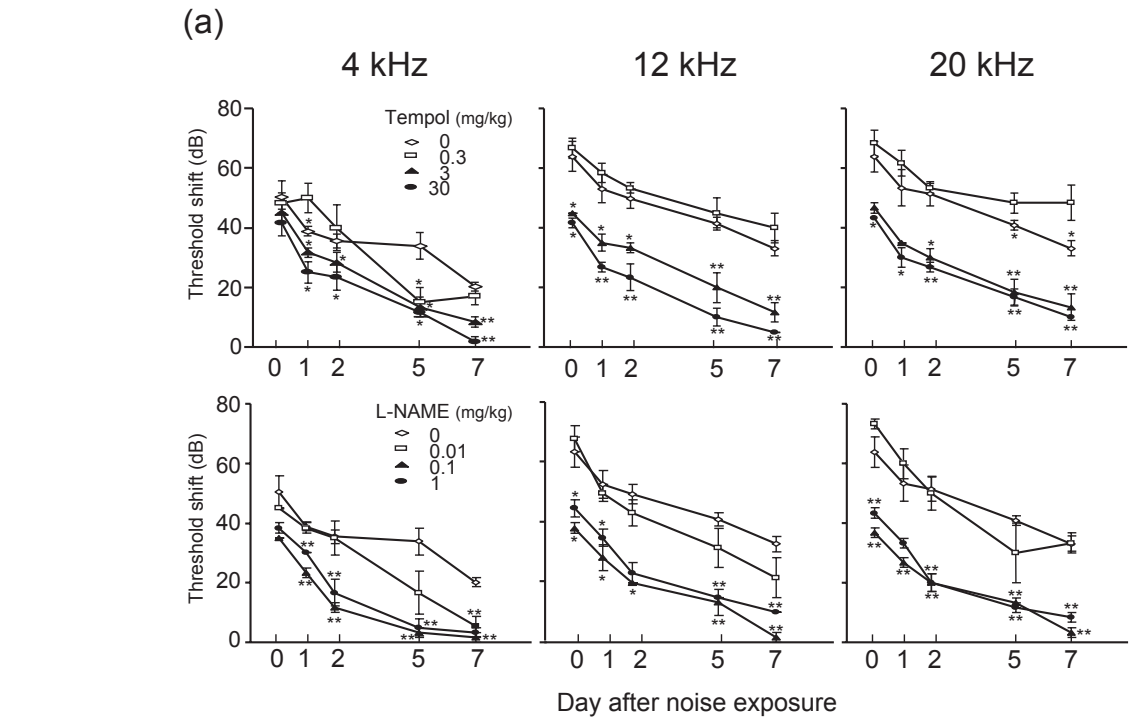

Fig. 4. Effect of tempol and L-NAME on ABR threshold shift and hair cell damage induced by noise exposure. Animals were exposed to noise at 110-dB SPL for 1 h. a) Tempol or L-NAME at the different doses indicated was intraperitoneally administered 30 min before the onset of noise exposure. The ABR threshold was assessed at the frequencies of 4, 12, and 20 kHz on days 0 (immediately), 1, 2, 5, and 7 after noise exposure. Graphs denote the average of threshold shift at each frequency. Values are the means ± S.E.M. from 4 independent experiments. $*P < 0.05$, $**P < 0.01$, significantly different from the value obtained for noise-exposed animals without drug treatment. b) Tempol (30 mg/kg) or L-NAME (1 mg/kg) was intraperitoneally administered 30 min before the onset of noise exposure. On day 14 after the exposure, animals were fixed for dissection of the organ of Corti, whose basal turn was histologically assessed by rhodamine-phalloidin staining. These experiments were carried out at least 3 times, with similar results obtained under the same experimental conditions.

wall immediately (Fig. 5b, time = 0) and extremely by 2 h post-exposure. However, the level of JNK was not significantly affected at any times post-exposure. The ratio of p-JNK to JNK was 2 and 5 immediately after and at 2 h post-exposure, respectively.

Figure 6 shows the effect of tempol and L-NAME on activation of JNK pathway–associated proteins following noise exposure at 110-dB SPL. Noise had the ability to increase the number of cells positive for p-SEK-1, p-JNK, p-c-Jun, and p-ATF-2 in both the spiral ligament and stria vascularis of the lateral wall at 2 h post-exposure. Either tempol or L-NAME produced a significant decrease in the number of cells positive for p-SEK-1, p-JNK, or p-c-Jun. The increase in the number of p-ATF-2 in the lateral wall of noise-exposed animals was at least in part abolished by tempol or L-NAME.

Effect of tempol and L-NAME on the decrease in the level of connexin26 in the spiral ligament following noise exposure

Non-sensory cells in the cochlea are connected extensively by gap junctions that facilitate intercellular ionic and biochemical coupling. Of the connexin family proteins, cinnexin26 and connexin30 are the prominent

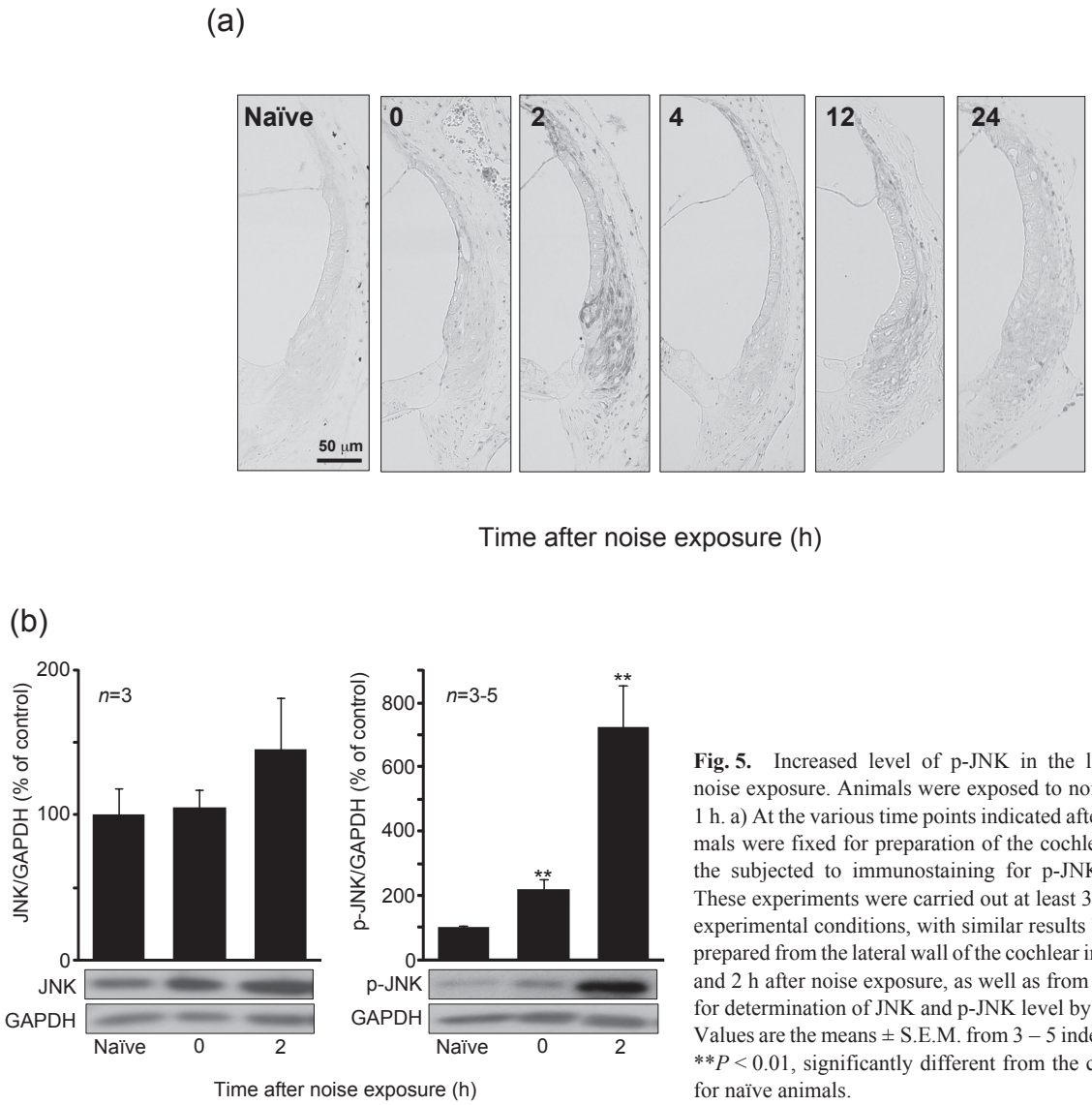

Fig. 5. Increased level of p-JNK in the lateral wall following noise exposure. Animals were exposed to noise at 110-dB SPL for 1 h. a) At the various time points indicated after noise exposure, animals were fixed for preparation of the cochlear slices, which were the subjected to immunostaining for p-JNK in the lateral wall. These experiments were carried out at least 3 times under the same experimental conditions, with similar results b) Homogenates were prepared from the lateral wall of the cochlear immediately (time = 0) and 2 h after noise exposure, as well as from that of naïve animals, for determination of JNK and p-JNK level by immunoblot analysis. Values are the means ± S.E.M. from 3 – 5 independent experiments. $^{**}P < 0.01$, significantly different from the control value obtained for naïve animals.

members co-assembled in most of the cochlear gap junctions (22). To evaluate whether connexin26 was involved in noise-induced events in the lateral wall, we assessed the connexin26 level in the lateral wall before and following noise exposure, with and without pretreatment with tempol and L-NAME (Fig. 7). Immunostaining revealed that most of the connexin26 was located in the spiral ligament, but not in the stria vascularis, of the lateral wall. The level of connexin26 was dramatically down-regulated at 4 h post-exposure. Expectedly, the noise-induced down-regulation of connexin26 was prevented by pretreatment with tempol or L-NAME (Fig. 7a). Quantitative analysis using immunoblot analysis showed noise exposure produced a dramatic decrease in the level of connexin26 in the lateral wall 4 h later, with tempol and L-NAME significantly preventing this decrease (Fig. 7b).

Decreased Na^+, K^+-ATPase activity in the lateral wall following noise exposure

It is well known that Na^+, K^+-ATPase in the spiral ligament participates in the active transport of Na^+ and K^+ for maintaining cochlear function (23). To evaluate involvement of Na^+, K^+-ATPase in noise-induced events in the lateral wall, we assessed the Na^+, K^+-ATPase activity in the lateral wall of naïve and noise-exposed animals. Interestingly, Na^+, K^+-ATPase activity was dramatically reduced at 2 h post-exposure [Na^+, K^+-ATPase activity (μmol·min^{-1}·mg protein^{-1}): naïve, 0.90 ± 0.33; noise, 0.09 ± 0.04 ($P < 0.05$ vs. naïve, n = 6)]. Expectedly, prior treatment with tempol (30 mg/kg) or L-NAME (1 mg/kg) was effective in decreasing the amount of noise-induced

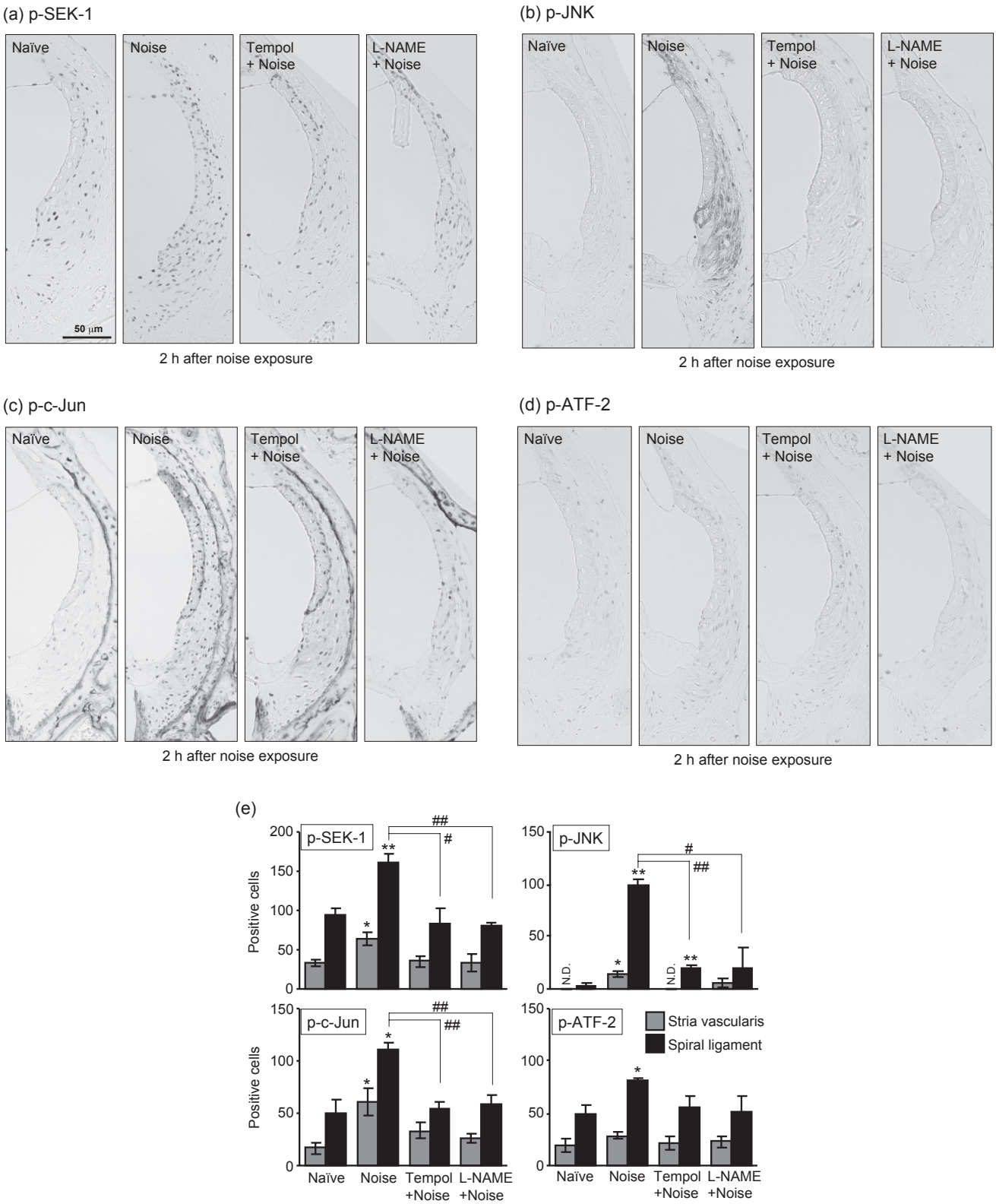

Fig. 6. Effect of tempol and L-NAME on noise-induced activation of SEK-1/JNK signals in the lateral wall. Animals were given tempol (30 mg/kg, i.p.) or L-NAME (1 mg/kg, i.p.) and then exposed to noise at 110-dB SPL for 1 h at 30 min after drug treatment. Sections were prepared for immunostaining of p-SEK-1 (a), p-JNK (b), p-c-Jun (c), and p-ATF-2 (d). e) Positive cells were counted separately in the spiral ligament and stria vascularis. Values are the means ± S.E.M. from 3 independent experiments. $*P < 0.05$, $**P < 0.01$, significantly different from the control value obtained for naïve animals. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, significantly different from the value obtained for noise-exposed animals without drug treatment.

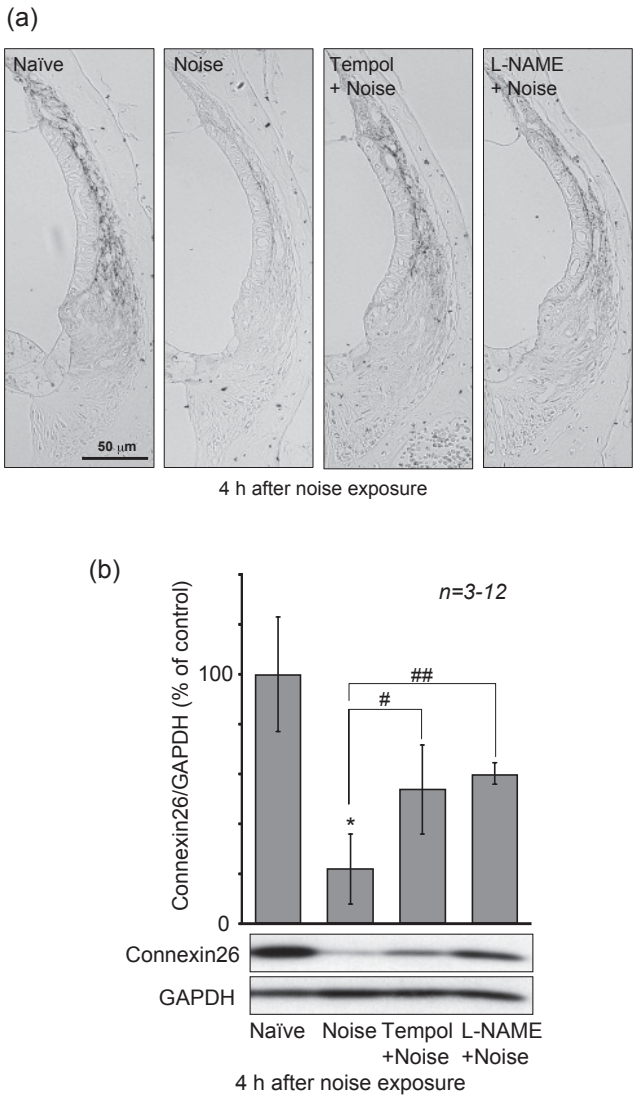

Fig. 7. Effect of tempol and L-NAME treatment on noise-induced decrease in connexin26 level in the lateral wall. Tempol (30 mg/kg) or L-NAME (1 mg/kg) was intraperitoneally administered 30 min before the onset of noise exposure at 110-dB SPL for 1 h. a) At 4 h after noise exposure, animals were fixed for preparation of the cochlear slices, which were subjected to immunostaining for connexin26. These experiments were carried out at least 3 times, with similar results under the same experimental conditions. b) For determination of connexin26 level by immunoblot analysis, homogenates were prepared from the lateral wall of the cochlear in noise-exposed animals and naïve animals. Values are the means ± S.E.M. from 3 – 12 independent experiments. $^{*}P < 0.05$, significantly different from the control value obtained for naïve animals. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, significantly different from the value obtained for noise-exposed animals without drug treatment.

reduction in Na^+, K^+-ATPase activity in the lateral wall [Na^+, K^+-ATPase activity (μmol·min^{-1}·mg protein^{-1}): noise + tempol, 0.54 ± 0.11 ($P < 0.01$ vs. noise alone, n = 6); noise + L-NAME, 0.30 ± 0.02 ($P < 0.01$ vs. noise alone, n = 6)].

Discussion

The primary finding of the present study is that the protective effect of the ROS scavenger and NOS inhibitor on acoustic injury in the cochlea is due to the prevention of noise-induced events such as the activation of the JNK pathway and down-regulation of connexin26 in the spiral ligament fibrocytes. Evidence that the present noise condition resulted in acoustic injury in the cochlea come from functional and histological assessments with ABR and rhodamine-phalloidin staining. The present noise condition produced at least a partial, but incomplete, permanent threshold shift and outer hair cell loss. It was confirmed by histological assessment that no damage was seen in the spiral ganglion cells, spiral ligament fibrocytes, and stria vascularis at least up to 30 days after the present noise exposure. Thus, the noise condition in the present study would be useful for evaluating the functional changes in the lateral wall during acoustic injury in the cochlea.

As stated earlier, evidence for noise-induced generation of ROS/NO in the cochlea comes from the findings that noise exposure increases the levels of hydroxyl radicals in cochlear fluids (2), superoxide anion radicals (24), and glutathione in the stria vascularis (25) and NO in the cochlear lateral wall (26). Previous studies on NOS in the cochlea demonstrated that whereas much less NOS activity is observed in the organ of Corti (27), nNOS (NOS1) and eNOS (NOS3) are located constitutively in the modiolar core and lateral wall, respectively (28). Furthermore, it was also previously demonstrated that acoustic overstimulation promotes the expression of iNOS (NOS2) in the spiral ligament (29). Our present finding that noise exposure increased nitrotyrosine immunoreactivity in spiral ganglion cells and spiral ligament fibrocytes, but not in the organ of Corti, supports the previous reports. Accumulating evidence suggests that ROS/NO is involved in hearing loss induced by intense noise and ototoxic drugs. The present data showing that tempol and L-NAME had the ability to protect against functional hearing impairment and hair cell loss induced by intense noise would be important for evaluating the mechanism underlying the protective effect of these drugs on acoustic injury.

4-HNE is a major aldehydic product of lipid peroxidation and believed to be largely responsible for the cytopathological effects observed during oxidative stress (19, 20). 4-HNE is largely responsible for many types of cellular damage associated with oxidative stress, having a high stability and reactivity to proteins and nucleotides. 4-HNE–protein adducts are detected in a number of disease lesions (30). 4-HNE also affects protein synthesis and several cell signaling pathways (31) by modulating

the activity of numerous enzymes such as GAPDH (32), glucose-6-phosphate dehydrogenase (33), glutathione reductase (34), interleukin-1β converting enzyme (35), and Na^+, K^+-ATPase (36). Of these enzymes, the ion-exchange enzyme Na^+, K^+-ATPase is known to participate in the active transport of Na^+ and K^+ in the inner ear and plays an important role in maintaining cochlear function (23, 37). In the present study, we obtained the first evidence for an increase in the level of an approximately 50 – 60 kDa 4-HNE–protein adduct in the lateral wall following noise exposure. These findings suggest the possibility that the function(s) of this protein in the lateral wall could be modulated by adduct formation with 4-HNE, which is generated by noise exposure. Indeed, the current study showed that Na^+, K^+-ATPase activity in the lateral wall was dramatically decreased by noise exposure, with it being recovered by tempol and L-NAME. These findings are evidence for ROS/NO-dependent impairment of the lateral wall in noise-exposed animals. Because evaluation of the mechanism underlying noise-induced decrease in Na^+, K^+-ATPase activity is lacking, future evaluation of whether this protein is modified by 4-HNE in the lateral wall following acoustic overstimulation could give us a hint for elucidating the cochlear events induced by acoustic insult.

NO and 4-HNE are known to cause activation of the JNK pathway, which is strongly regulated by various forms of stress and believed to play an important role in apoptosis (38, 39). Here we provided the first demonstration that noise exposure activated the JNK pathway in the murine cochlear spiral ligament fibrocytes. Evidence for involvement of this pathway in acoustic injury comes from previous reports showing that the JNK pathway in the organ of Corti is associated with noise trauma–induced injury and apoptosis of the hair cells (40) and that a peptide inhibitor of JNK protects against both aminoglycosides and acoustic trauma–induced auditory hair cell death and hearing loss (41). In addition to these earlier reports, the present results allow us to propose the idea that in the spiral ligament fibrocytes, activation of the JNK pathway in the fibrocytes of the spiral ligament was directly or indirectly involved in acoustic injury; that is., our present findings show that the activation of JNK was completely abolished by pretreatment with tempol or L-NAME, which protected against hearing impairment induced by acoustic overstimulation.

The expression levels of connexin proteins, gap junction components, often regulate cell–cell interactions in numerous cells (42). In the cochlea, it is known that non-sensory cells are connected extensively by gap junctions that facilitate intercellular ionic and biochemical coupling. More interestingly, our present study has provided the first data demonstrating a decreased level of connexin26 in the spiral ligament fibrocytes following noise exposure, whose decrease was prevented by tempol and L-NAME. Hence, it is most likely that the decrease in connexin26 level is due to excess ROS/NO generated by acoustic overstimulation. However, there is no evidence for participation of JNK signaling in the downregulation in the present study. Non-sensory cells in the cochlea are connected extensively by gap junctions that facilitate intercellular ionic and biochemical coupling. Two protein subunits in the connexin family, connexin26 and connexin30, are the prominent members co-assembled in most of the cochlear gap junction (22). Connexin26 is mainly responsible for cationic and anionic permeability in the cochlear sensory epithelium and plays an important role in the intercellular signaling. Mutations in the gene coding for connexin26 are also known to cause a substantial portion of human nonsyndromic hereditary deafness cases, resulting in one of the most common human birth defects. Therefore, the possibility that the observed noise-induced decrease in connexin26 protein produces a loss of ionic balance in the cochlea followed by functional hearing impairment is feasible.

In summary, prior treatment with tempol and L-NAME attenuated the ABR threshold shift and hair cell loss induced by intense noise, with a concomitant abolishment of noise-induced activation of the JNK pathway in the spiral ligament fibrocytes. In addition, both drugs were capable of preventing conexin26 from being down-regulated in the spiral ligament fibrocytes following intense noise exposure. As our hypothesis, acoustic overstimulation in the cochlea generates ROS and NO/peroxynitrite in the spiral ligament fibrocytes, where these radicals at least the activate the JNK pathway. In addition to this activation, the expression of connexin26 in the spiral ligament is negatively regulated by ROS and NO/peroxynitrite generated by acoustic overstimulation. Thus, acoustic injury may be due, at least in part, to down-regulation of connexin family proteins in the spiral ligament. To elucidate the exact mechanisms underlying acoustic injury, we plan to study gap junction–associated proteins in the spiral ligament in the future.

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