Research paper

JNK signaling in neomycin-induced vestibular hair cell death

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Abstract

Mechanosensory hair cells are susceptible to apoptotic death in response to exposure to ototoxic drugs, including aminoglycoside antibiotics. The c-Jun n-terminal kinase (JNK) is a stress-activated protein kinase that can promote apoptotic cell death in a variety of systems. Inhibition of the JNK signaling pathway can prevent aminoglycoside-induced death of cochlear and vestibular sensory hair cells. We used an *in vitro* preparation of utricles from adult mice to examine the role of JNK activation in aminoglycoside-induced hair cell death. CEP-11004 was used as an indirect inhibitor of JNK signaling. Immunohistochemistry showed that both JNK and its downstream target c-Jun are phosphorylated in hair cells of utricles exposed to neomycin. CEP-11004 inhibited neomycin-induced phosphorylation of both JNK and c-Jun. CEP-11004 inhibited hair cell death in utricles exposed to moderate doses of neomycin. However, the results were not uniform across the dose–response function; CEP-11004 did not inhibit hair cell death in utricles exposed to high-dose neomycin. The CEP-11004-induced protective effect was not due to inhibition of PKC or p38, since neither Chelerythrine nor SB203580 could mimic the protective effect of CEP-11004. In addition, inhibition of JNK inhibited the activation of caspase-9 in hair cells. These results indicate that JNK plays an important role in neomycin-induced vestibular hair cell death and caspase-9 activation.
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1. Introduction

Auditory and vestibular sensory hair cells are susceptible to damage from certain therapeutic drugs, including the aminoglycoside antibiotics and the antineoplastic agent cisplatin. Ototoxic drug-induced hair cell death has been characterized as apoptotic by many authors, based on both morphologic and molecular indicators (Li et al., 1995; Lang and Liu, 1997; Vago et al., 1998; Torchinsky et al., 1999; Forge and Li, 2000; Cunningham et al., 2002; Matsui et al., 2002; Cheng et al., 2003; Matsui et al., 2003; Mangiardi et al., 2004). Perhaps the most compelling evidence supporting this conclusion is that both cisplatin-induced and aminoglycoside-induced hair cell death are significantly inhibited by broad-spectrum inhibition of caspases (Liu et al., 1998; Cunningham et al., 2002; Matsui et al., 2002, 2003; Cheng et al., 2003; Shimizu et al., 2003).

The c-Jun NH$_2$-terminal kinases (JNKs), also known as "stress-activated protein kinases" (SAPKs), are in the family of mitogen-activated protein kinases (MAP kinases) that also includes extracellular signal-related kinase (ERK) and p38. These kinases are activated in cells in response to a variety of environmental stresses, including exposure to inflammatory cytokines, osmotic stress, radiation, and excitotoxicity (reviewed by Ip and Davis, 1998). JNKs are activated by dual phosphorylation of Thr and Tyr residues by MAP kinase kinases (MKKs). JNK in turn phosphorylates c-Jun, a component of the transcription factor complex AP-1 (Whitmarsh and Davis, 1996). AP-1 is composed of members of the bZIP group of DNA proteins (c-Fos,

Fos B, Fra-1, Fra-2, c-Jun, Jun B, and Jun D), which form homo- and heterodimers with one another. These dimers recognize and bind to a specific DNA sequence, the AP-1 site (5′-TGACTCA-3′) (Ham et al., 2000). The phosphorylation of transcriptional components by JNK leads to increased AP-1 activity. AP-1 transcriptional activity is important for neuronal activation of pro-apoptotic members of the Bcl-2 family (Harris and Johnson, 2001; Whitfield et al., 2001).

JNK activation has also been shown to play an important role in aminoglycoside-induced hair cell death. In cochlear explants from neonatal rats, exposure to neomycin results in phosphorylation of both JNK and c-Jun (Pirvola et al., 2000). CEP-1347 is a small-molecule inhibitor of the family of mixed-lineage kinases (MLKs) (Maroney et al., 2001). MLKs are MAP kinase kinase kinases (MKKKs) that function upstream of the MAP kinase kinases (MKKs) that activate JNKs and other MAP kinases (Maroney et al., 2001). Pirvola et al. (2000) tested the ability of CEP-1347 to protect hair cells against aminoglycoside ototoxicity and noise trauma. In cochlear explants from P2 rat pups, neomycin exposure resulted in death of >80% of hair cells in the basal turn. However, co-incubation with CEP-1347 protected >90% of the hair cells from neomycin-induced apoptosis (Pirvola et al., 2000). CEP-1347 also attenuates gentamicin-induced hair cell death and hearing loss *in vivo* (Ylikoski et al., 2002). Additional evidence for an important role for JNK in ototoxic hair cell death comes from experiments using a peptide inhibitor of JNK, termed D-JNK-1. This peptide blocks JNK-mediated phosphorylation of c-Jun (Bonny et al., 2001). In organ of Corti explants from P3 mice, D-JNK-1 prevented over 90% of the hair cell death caused by exposure to neomycin (Wang et al., 2003). Another inhibitor of mixed lineage kinases, CEP-11004, inhibits aminoglycoside-induced hair cell death in chick utricles (Matsui et al., 2004) and neonatal rat organ of Corti explants (Bodmer et al., 2002). Here we have examined the effect of CEP-11004 on neomycin-induced hair cell death in the adult mouse utricle *in vitro*. We have also determined the range of aminoglycoside doses across which inhibition of JNK is effective in inhibiting hair cell death. In addition, we have examined the molecular pathway through which JNK activation functions in hair cell death. Finally, we have examined the effects of inhibition of two other cellular protein kinases, p38 and protein kinase C (PKC) on neomycin-induced hair cell death.

2. Materials and methods

2.1. Animal use and care

CBA/CaJ mice were obtained from The Jackson Laboratory (Bar Harbor, ME). All mice (4–6 weeks of age) were male and had normal Preyer's reflexes. All experimental protocols were reviewed and approved by the University of Washington Animal Care and Use Committee.

2.2. Organ culture of utricles and induction of hair cell death

All animals were killed by an overdose of pentobarbital and immediately decapitated. The temporal bones were quickly removed and the individual vestibular organs were dissected in medium consisting of Basal Medium Eagle (BME, Invitrogen, Carlsbad, CA) supplemented with Earle's Balanced Salt Solution (EBSS, Invitrogen) (2:1, v/v). Utricles were then immediately moved into the culture medium, which consisted of BME supplemented with EBSS (2:1, v/v) and 5% fetal bovine serum (Invitrogen). The utricles were incubated (submerged but not adhered) in 24 well tissue culture plates for 12 or 24 h at 37 °C in a 5% CO_2 and 95% air environment (Forma Scientific, Marietta, OH). Neomycin solution (10 mg/ml; Sigma, St. Louis, MO) was added into the culture wells at final concentrations ranging from 1.0 to 4.0 mM. Neomycin was not added to the cultures in the control group. At the end of the culture period, utricles were fixed with 4% paraformaldehyde for 1 h at room temperature (RT). Otoconia were gently removed from fixed utricles with a stream of PBS applied via 28 G needle and syringe. The culture methods for the mouse utricle preparation have been described in detail (Cunningham, 2006).

2.3. Immunohistochemistry for hair cell labeling

Fixed utricles were incubated in 1% H_2O_2 in PBS for 30 min at RT to quench endogenous peroxidases. Samples were then incubated in blocking solution (1% bovine serum albumin, 0.4% normal goat serum, 0.4% normal horse serum, 0.4% Triton X-100 in PBS) for 3 h at RT. For hair cell counts, utricles were double-labeled in whole mount using a monoclonal antibody against calmodulin (Sigma # C-3545) and a polyclonal antibody against calbindin (Chemicon # AB1778, Temecula, CA). This double-label immunochemistry protocol allows for separate counts of hair cells in the extrastriolar (calmodulin positive, calbindin negative) and striolar (calmodulin and calbindin positive) regions (Cunningham et al., 2002; Cunningham, 2006). Samples were incubated overnight at 4 °C in primary antibody solution (calmodulin 1:150, calbindin 1:250 in blocking solution). After washing with blocking solution, the specimens were incubated in secondary antibodies diluted in blocking solution as follows: biotinylated horse anti-mouse IgG (1:100; Vector Laboratories, Burlingame, CA) or Alexa 488 conjugated goat anti-mouse IgG (1:500; Molecular Probes, Eugene, OR) in addition to Alexa 594 conjugated goat anti-rabbit IgG (1:500; Molecular Probes). After rinsing with blocking solution, the utricles that had been incubated with biotinylated secondary antibody were incubated for 2 h in avidin–biotin horseradish peroxidase complex (Vector Laboratories) for labeling using peroxidases. The tissue was rinsed in 0.05 M of Tris buffer, pH 7.4, and placed in diaminobenzidine (0.375 mg/ml in 0.05 M of Tris buffer and 0.1% of H_2O_2) for 10 min at RT. Utricles that were incubated with fluorescent secondary antibodies

were washed in blocking solution. The utricles were mounted in Fluoromount-G (Electron Microscopy Sciences, Washington, PA) and coverslipped.

2.4. Phosphorylated c-jun and phosphorylated JNK labeling

Fixed utricles were incubated in blocking solution (1% bovine serum albumin, 0.4% normal goat serum, 0.4% normal horse serum, 0.4% Triton X-100 in PBS) for 3 h at RT. Samples were then incubated with either a rabbit polyclonal antibody specific for c-Jun that is phosphorylated on serine 63 (1:100; # 9261 Cell Signaling Technology, Beverly, MA), or a rabbit polyclonal antibody specific for JNK that is dually phosphorylated on threonine 183 and tyrosine 185 (1:100; # 9251 Cell Signaling Technology) at 4 °C overnight. The monoclonal antibody against calmodulin (1:150; Sigma) was added to the primary antibody solution in order to label hair cells. After washing with blocking solution, samples were incubated for 4 h at RT in the secondary antibody solution as follows: Alexa 594 conjugated goat anti-rabbit IgG (1:500; Molecular Probes) and Alexa 488 conjugated goat anti-mouse IgG (1:500; Molecular Probes). After rinsing in blocking solution, samples were whole-mounted in Fluoromount-G and coverslipped.

2.5. Evaluation of caspase-9 activation

The fluorescent caspase-9 substrate fam-LEHD-fmk (CaspaTag™, Intergen Co., Purchase, NY) was used to detect the activity of caspase-9. The substrate was dissolved in dimethyl sulfoxide (DMSO) to 150× (stock) solution. Aliquots of this solution were stored at −20 °C in the dark. Prior to use, 30× working solution was prepared by diluting the stock solution 1:5 in PBS. Utricles were cultured in the presence or absence of neomycin (1 mM) for 11 h. For the final (12th) hour in culture, 20 µl of 30× caspase-9 substrate solution was added to the wells of the tissue culture plate containing the utricles. Utricles were then incubated with the fluorescent substrate for 1 h under the same conditions. After incubation, the utricles were rinsed three times at 37 °C with the wash buffer supplied by the manufacturer. The tissues were fixed overnight at 4 °C with the fixative supplied by the manufacturer. After fixation and removal of otoconia, the utricles were incubated for 2 h at RT in rhodamine-conjugated phalloidin (Molecular Probes) (1:100 in PBS containing 0.05% Triton X-100). After rinsing in PBS, samples were whole-mounted in Fluoromount-G. Activated caspase-9 was visualized with a confocal laser-scanning microscope (MRC-1024; Bio-Rad, Hercules, CA). In order to ensure that only hair cells were examined, the depth of focus of z-series confocal micrographs was limited to 5 µm below the stereocilia bundles. The images were processed digitally with ImageJ (NIH, Bethesda, MD) and Photoshop (Adobe, Mountain View, CA). Since there were fewer than 100 caspase-9-positive hair cells in each utricle, we counted all of them and reported the number as total number of positive hair cells/utricle.

2.6. Inhibition of JNK signaling

CEP-11004, an inhibitor of mixed-lineage kinases (MLKs) was used as an indirect inhibitor of JNK activation. CEP-11004 was generously provided by Cephalon (Fraser, PA, USA). CEP-11004 was dissolved in DMSO and stored at −20 °C as a 1 mM stock solution. The inhibitor was diluted in culture medium (final concentrations 0.1–2.0 µM) before each culture and applied to each tissue culture well 4 h before addition of neomycin. To examine the effects of inhibition of p38 MAP kinase and PKC, SB203580 (10 µM, Calbiochem, La Jolla, CA) and Chelerythrine (13 µM, ALEXIS Inc., San Diego, CA) were added to some cultures.

2.7. Hair cell counts

Utricles were examined on a Leitz (Wetzlar, Germany) Aristoplan upright microscope to evaluate the survival of hair cells. Calbindin-positive and calmodulin-positive cells were counted as the hair cells in the striolar region and extrastriolar region, respectively. The labeled hair cells were counted in each of eight randomly selected areas (squares 30 µm on a side in each utricle). The four striolar and four extrastriolar hair cell counts were each averaged to produce one striolar and one extrastriolar hair cell density for each utricle examined. At least five utricles were examined for each experimental condition.

2.8. Statistical analyses

One-way ANOVA with Newman–Keuls *post hoc* test was performed using GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego California. Two-way ANOVA with Tukey's post hoc tests was performed using SYSTAT version 8.0 for Windows, SPSS Software, Chicago, IL, 1998.

3. Results

3.1. The MLK inhibitor CEP-11004 suppresses JNK signaling

To detect the activation of JNK in hair cells, immunohistochemistry was performed using phospho-specific antibodies directed against phosphorylated forms of both JNK and c-Jun, which is a downstream target of JNK. We had previously shown that caspase activation peaks at 12 h in neomycin-exposed hair cells in this preparation (Cunningham et al., 2002). Therefore, utricles were cultured for 12 h in 1 mM neomycin. Hair cells were immunolabeled with anti-calmodulin. Representative results are shown in Fig. 1. In control utricles (cultured without neomycin), few (or no) hair cells with immunoreactivity for phosphorylated JNK or c-Jun were detected (Fig. 1A and D). Many hair cells with phosphorylated JNK and c-Jun were present in the utricles cultured with neomycin (Fig. 1B and E). Indirect inhibition of JNK activation using 1.0 µM CEP-11004
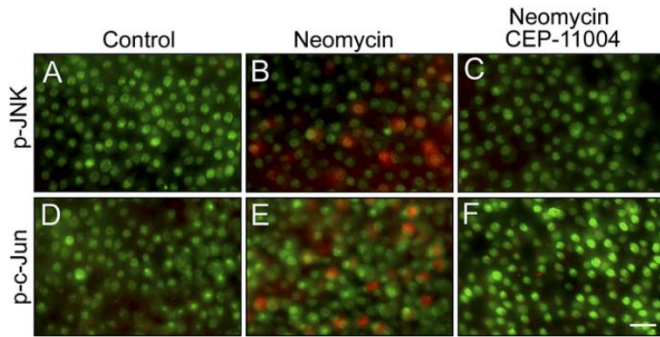
Fig. 1. CEP-11004 inhibits phosphorylation of both JNK and c-Jun. Utricles were cultured for 12 h without neomycin (A, D), with 1 mM neomycin (B, E), or with both neomycin and CEP-11004 (C, F). Hair cells were labeled with anti-calmodulin (green). Phosphorylated JNK (A–C) and c-Jun (D–F) were detected using phospho-specific antibodies (red). Neomycin exposure resulted in phosphorylation of both JNK (B) and c-Jun (E) in hair cells. CEP-11004 inhibited neomycin-induced phosphorylation of both JNK (C) and c-Jun (F). Scale bar in $F = 10\,\mu m$.

appeared to suppress neomycin-induced phosphorylation of both JNK and c-Jun (Fig. 1C and F).

3.2. JNK inhibition protects hair cells against neomycin-induced death

To evaluate the role of JNK signaling in neomycin-induced hair cell death, utricles were cultured with a moderate dose of neomycin (1 mM) and varying doses of CEP-11004 (0–2.0 μM) for 24 h. Utricles were fixed and prepared for hair cell counts using calmodulin and calbindin double-labeling. Results are shown in Fig. 2. Utricles cultured in the absence of neomycin showed normal hair cell densities in both the striolar and extrastriolar regions (Fig. 2A and E). Hair cell survival was reduced significantly in utricles cultured with neomycin (1 mM) for 24 h (Fig. 2B and F). CEP-11004 inhibited neomycin-induced hair cell death (Fig. 2C, G and I, two-way ANOVA, $p < 0.0001$). The most effective concentration of CEP-11004 was 1.0 μM (Fig. 2C, G and I), having a significant effect ($p < 0.01$) on both striolar and extrastriolar hair cells. At this concentration, hair cell densities were 92% and 81% of control values for the extrastriolar and striolar regions, respectively, despite the presence of neomycin. CEP-11004 also had a small but reliable protective effect at 0.5 μM ($p < 0.05$) in the extrastriolar region. In the striolar region, CEP-11004 was not effective at 0.5 μM ($p > 0.05$). These data are consistent with our previous finding that the striolar hair cells are more susceptible to neomycin-induced hair cell death and also more difficult to protect (Cunningham et al., 2002). At other concentrations (2 μM, 0.2 μM, 0.1 μM), CEP-11004 did not significantly inhibit neomycin-induced hair cell death ($p > 0.05$, Fig. 2D, H and I).

In order to determine the range of neomycin doses across which JNK inhibition protects hair cells, we used the most effective dose of CEP-11004 (1 μM) in conjunction with neomycin doses that span the entire dose–
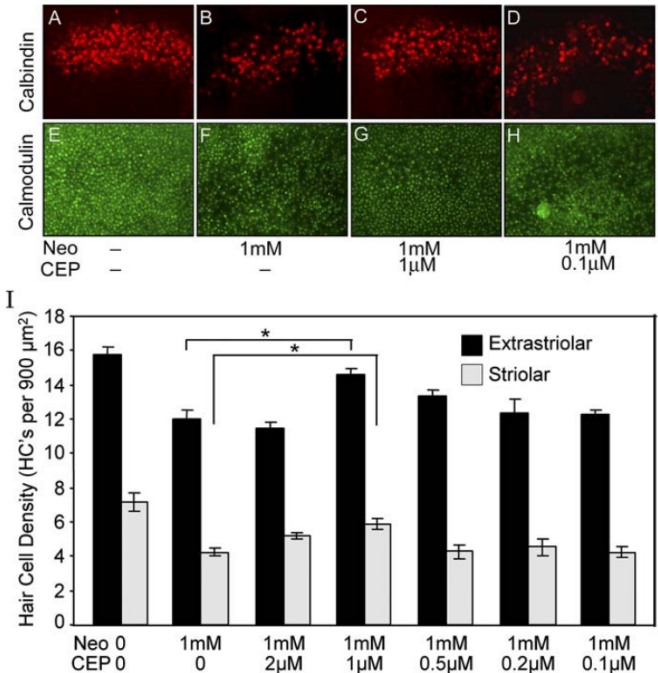

Fig. 2. CEP-11004 inhibits neomycin-induced hair cell death. Utricles were cultured for 24 h without neomycin (A, E), with 1 mM neomycin (B, F), or with both neomycin and CEP-11004 (C, G, D, H). Utricles were double-labeled for calmodulin (green) and calbindin (red) immunoreactivity. Control utricles appeared normal and had normal numbers of hair cells in both the striolar (A) and extrastriolar (E) regions. Neomycin (B, F) resulted in significant hair cell death, particularly in the striolar region. At a concentration of 1 μM, CEP-11004 inhibited neomycin-induced death of both striolar (C) and extrastriolar (G) hair cells. CEP-11004 had no protective effect at 0.1 μM (D, H). Results are quantified in panel I: Hair cells were counted in calmodulin/calbindin double-labeled utricles. CEP-11004 significantly inhibited hair cell death (two-way ANOVA, $p < 0.0001$). CEP-11004 was most protective at 1 μM (Tukey's post hoc test, $p < 0.01$ for both the striolar and extrastriolar regions). At 0.5 μM, CEP-11004 was significantly protective only in the extrastriolar region (Tukey's post hoc test, $p < 0.05$ for the extrastriolar region, $p > 0.1$ for the striolar region). Both higher (2 μM) and lower (0.2 μM, 0.1 μM) concentrations of CEP-11004 failed to significantly inhibit neomycin-induced hair cell death. Shown are mean ($\pm$SEM) hair cell densities for both the striolar and extrastriolar regions for $n = 5$–8 utricles per experimental condition. Asterisks indicate significance (Tukey's post hoc test $p < 0.05$).

response relationship (Cunningham et al., 2002). Results are shown in Fig. 3. In the extrastriolar region, CEP-11004 reliably inhibited neomycin-induced hair cell death at doses near the middle of the dose–response relationship (1 mM and 2 mM). However, CEP-11004 did not have a significant protective effect against hair cell death in response to high-dose (4 mM) neomycin. In the striolar region, CEP-11004 inhibited hair cell death only at 1 mM neomycin and not at any of the other doses tested (data not illustrated).

Since CEP-11004 is an indirect inhibitor of JNK signaling, we did two additional experiments to confirm that the protective effects we observed with CEP-11004 were due to inhibition of JNK. At high concentrations CEP-11004 can bind to PKC (Murakata et al., 2002). To confirm that the protective effect of CEP-11004 on hair cells was attributable
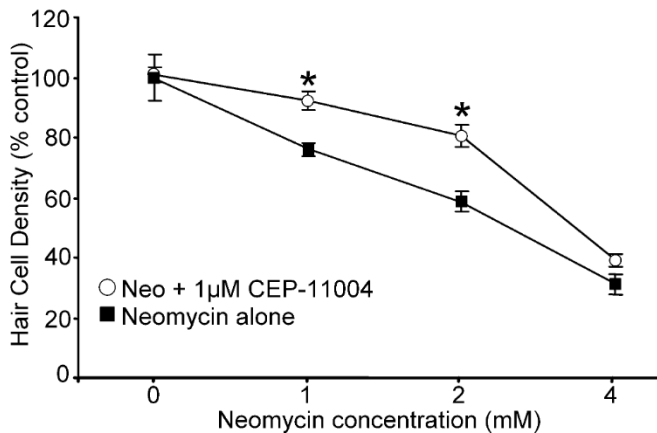
Fig. 3. CEP-11004 is protective against moderate doses of neomycin. Utricles were cultured for 24 h in the presence (or absence) of neomycin alone or neomycin plus 1.0 µM CEP-11004. Shown are hair cell densities for the extrastriolar region. CEP-11004 significantly inhibited hair cell death when the neomycin dose was near the middle of the dose–response curve (1 mM and 2 mM neomycin, two-way ANOVA, $p < 0.001$). CEP-11004 was not effective at inhibiting hair cell death in response to high-dose neomycin (4 mM). In the striolar region, the protective effect of CEP-11004 was significant only at 1 mM neomycin (data not shown). Shown are normalized hair cell densities (mean ± SEM) for $n = 5$–8 utricles per condition. Asterisks indicate significance (Tukey's post hoc tests, $p < 0.001$).

to inhibition of JNK and not PKC, we examined whether the PKC inhibitor Chelerythrine could mimic the effects of CEP-11004. Inhibition of PKC with 13 µM Chelerythrine did not inhibit neomycin-induced hair cell death (Fig. 4). In fact, Chelerythrine appeared to potentiate the effects of neomycin, at least in the extrastriolar region, although incubation of utricles with Chelerythrine alone did not
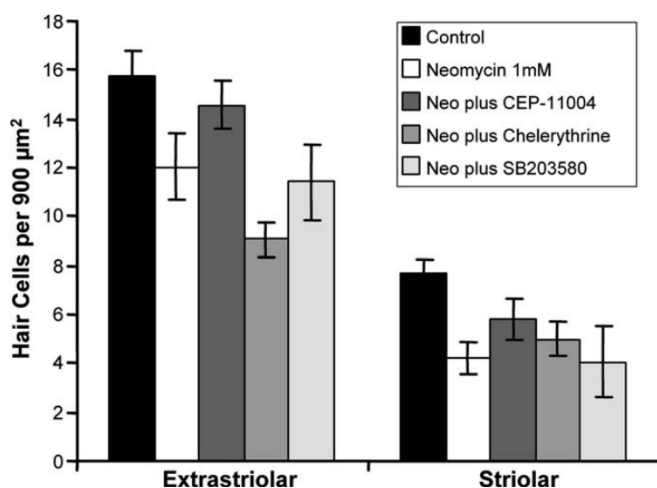

Fig. 4. Neomycin-induced hair cell death is not inhibited by inhibition of PKC or p38. Utricles were cultured for 24 h in control conditions, neomycin alone (1 mM), or neomycin plus either CEP-11004, 13 µM Chelerythrine (an inhibitor of PKC), or 10 µM SB203580 (an inhibitor of p38). Shown are hair cell densities for both the extrastriolar and striolar regions. Neither Chelerythrine nor SB203580 showed a protective effect against neomycin-induced hair cell death. Shown are mean ± SEM hair cell densities for $n = 5$–8 utricles per condition.

result in hair cell death (data not illustrated). An additional experiment was aimed at determining whether inhibition of p38 MAP kinase could mimic the effects of CEP-11004. CEP-11004 is an inhibitor of mixed lineage kinases (MLKs), which are upstream activators of JNK. Here we used CEP-11004 to inhibit JNK phosphorylation and activation. However, MLKs can also regulate signaling by the p38 MAP kinase in addition to JNK (Harper and LoGrasso, 2001; Gallo and Johnson, 2002). Therefore, the effect of inhibition of p38 MAP kinase on ototoxic hair cell death was investigated using the p38 kinase inhibitor SB203580 (Cuenda et al., 1995). Inhibition of p38 did not significantly affect neomycin-induced hair cell death (Fig. 4). These data suggest that the protective effect of CEP-11004 occurs via inhibition of JNK signaling and not via inhibition of either PKC or p38 MAP kinase.

3.3. JNK inhibition supresses neomycin-induced caspase-9 activation

Aminoglycoside-induced hair cell death is mediated by caspases, specifically caspase-9 (Cunningham et al., 2002; Matsui et al., 2003; Cunningham et al., 2004; Lee et al., 2004). Therefore, we examined the relationship between JNK activity and caspase-9 activation. Utricles were cultured for 12 h in the presence (or absence) of 1 mM neomycin, and caspase-9 activation was measured using the
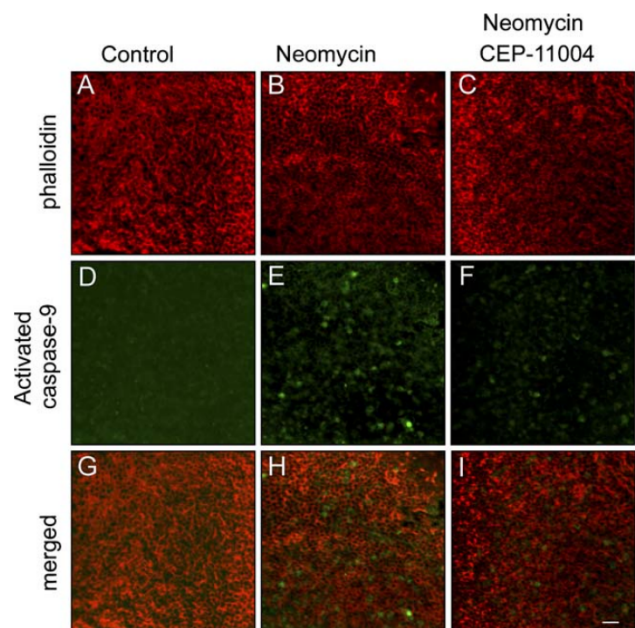

Fig. 5. CEP-11004 inhibits neomycin-induced caspase-9 activation. Utricles were cultured for 12 h without neomycin (A, D, G), with 1 mM neomycin (B, E, H), or with both neomycin and CEP-11004 (C, F, I). Caspase-9 activation was examined using the fluorescent caspase-9 substrate FAM-LEHD-fmk (green). Hair cell stereocilia were labeled using rhodamine phalloidin (red). Very little caspase-9 activation is detected in the control utricles (A, D, G). Robust caspase-9 activation is present in utricles cultured in neomycin alone (B, E, H). CEP-11004 inhibits neomycin-induced activation of caspase-9 (C, F, I). Scale bar in $I = 10$ µm.
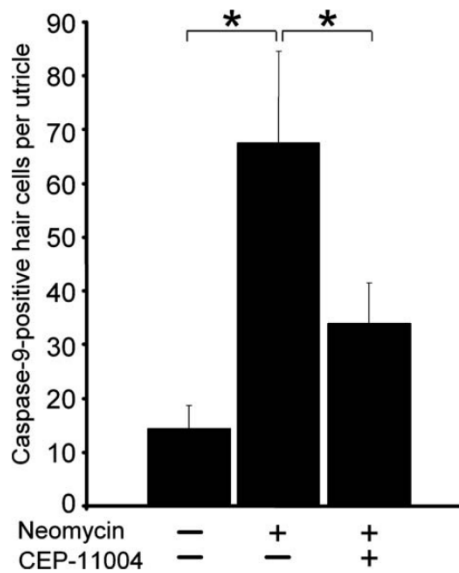
Fig. 6. Quantification of CEP-11004 inhibition of neomycin-induced caspase-9 activation. Caspase-9-positive hair cells were counted in utricles that were prepared as described in Fig. 5. Utricles cultured in control conditions showed few hair cells with activated caspase-9. Neomycin exposure resulted in robust (but variable) activation of caspase-9. CEP-11004 inhibited neomycin-induced caspase-9 activation in hair cells. Shown are mean ± SEM caspase-9 positive hair cells for $n = 5$–6 utricles per condition. Asterisk indicates significance (one-way ANOVA with Newman–Keuls post hoc test, $p < 0.05$).

fluorescent caspase-9 substrate FAM-VAD-fmk. Hair cell stereocilia bundles were labeled using rhodamine phalloidin. Fig. 5 shows representative results. Very little activated caspase-9 was detected in utricles cultured in the absence of neomycin (Fig. 5A, D and G). In contrast, robust activation of caspase-9 could be readily detected in the hair cells of utricles cultured in the presence of neomycin (Fig. 5E and H). CEP-11004 inhibited the neomycin-induced activation of caspase-9 in hair cells (Fig. 5F and I). These data were quantified by counting hair cells with activated caspase-9 (Fig. 6). The number of hair cells with activated caspase-9 was significantly lower in the utricles cultured with both neomycin and CEP-11004 than in those cultured with neomycin alone (Fig. 6, one-way ANOVA, $p < 0.05$). These results indicate that activated JNK is an upstream regulator of caspase-9 activation in hair cells.

4. Discussion

In this study, we examined the role of JNK signaling in neomycin-induced hair cell death in the mouse utricle *in vitro*. Reports on both neuronal and non-neuronal systems have indicated that JNK has an important role in stress-induced apoptosis. For example, *in vitro* studies have shown that JNK is required for NGF withdrawal-induced apoptosis of PC12 cells (Xia et al., 1995). Systemic administration of a peptide inhibitor of JNK protects cortical neurons against ischemia-induced apoptosis (Borsello et al., 2003). Disruption of the gene encoding JNK3 results in

resistance to excitotoxic stress-induced apoptosis of hippocampal neurons (Yang et al., 1997). Fibroblasts from mice lacking JNK activity are resistant to apoptosis induced by UV irradiation (Tournier et al., 2000). Absence of JNK in these fibroblasts prevents release of mitochondrial cytochrome c, indicating that JNK is required for mitochondrial activation of apoptosis (Tournier et al., 2000).

There have been several reports indicating that inhibition of JNK protects hair cells against aminoglycoside-induced death (Pirvola et al., 2000; Bodmer et al., 2002; Ylikoski et al., 2002; Wang et al., 2003; Matsui et al., 2004). Inhibition of JNK signaling also showed a protective effect against aminoglycoside-induced hair cell death in the present study using cultured mouse utricles. It is important to note that the protective effect of JNK inhibition is limited to a narrow range of neomycin doses, indicating that a severe insult can result in JNK-independent hair cell death. If data on molecules that inhibit aminoglycoside-induced hair cell death are to be translated into clinical therapies, it is important that these molecules show a protective effect across a broad range of aminoglycoside doses (Cheng et al., 2005). Thus, it appears that while data on the role of JNK activation may be vital to our understanding of the mechanisms underlying aminoglycoside-induced hair cell death, the translation of these data into clinical therapies may be limited by the range of aminoglycoside doses over which JNK inhibition is protective.

We examined the effects of inhibition of two additional protein kinases, p38 MAP kinase and PKC. Like JNK, p38 kinase is known to be activated by extracellular stress. The activation of p38 kinase occurs in response to some of the same signals that result in JNK activation (Harper and LoGrasso, 2001). MLKs are MAPKKKs that are upstream regulators of both JNK and p38 kinase (Gallo and Johnson, 2002). CEP-11004 is an inhibitor of MLKs, which could therefore influence signaling by p38 kinase in addition to JNK. To examine whether the protective effect of CEP-11004 on hair cells could be attributable to inhibition of p38 kinase, we tested whether specific inhibition of p38 protected hair cells against neomycin-induced death. We used the p38 kinase inhibitor SB203580 at 10 μM, a concentration which has been shown to be effective in preventing ceramide-induced death of cortical neurons (Willaime-Morawek et al., 2003). Inhibition of p38 did not demonstrate a protective effect against neomycin-induced hair cell death. In addition to its potential to effect p38 signaling, CEP-11004 has a weak affinity for PKC (Murakata et al., 2002). However, our experiments with Chelerythrine indicate that inhibition of PKC does not inhibit neomycin-induced hair cell death. Taken together, these data suggest that the protective effect of CEP-11004 is mediated by inhibition of JNK signaling and not by inhibition of p38 or PKC signaling.

While systemic administration of a JNK inhibitor reduces both cochlear hair cell loss and auditory threshold shift in mature animals exposed to aminoglycosides (Ylikoski et al., 2002; Wang et al., 2003), it has proven difficult to

detect JNK phosphorylation in cochlear hair cells using immunohistochemical methods (Ylikoski et al., 2002; Jiang et al., 2005). It has been suggested that this difficulty may be due to the decalcification procedures required to perform immunochemistry on adult cochlear tissues (Ylikoski et al., 2002). The lack of detectable JNK phosphorylation *in vivo* may also be reflective of the timing of JNK activation. If JNK remains phosphorylated for only a short period of time, it may be more easily detected in an *in vitro* model system, in which tissue can be more easily collected every few hours, than in an *in vivo* system where tissue collection timepoints may be separated by days. Still, it remains possible that JNK is not strongly phosphorylated in cochlear hair cells exposed to aminoglycosides, although inhibition of JNK clearly protects these cells *in vivo* (Ylikoski et al., 2002; Wang et al., 2003).

Aminoglycoside-induced JNK activation may be caused by generation of reactive oxygen species (ROS), which are rapidly formed in hair cells exposed to aminoglycosides (Priuska and Schacht, 1995; Hirose et al., 1997; Sha and Schacht, 1999). Oxidative stress can trigger activation of the redox-sensitive kinase ASK1, which in turn activates both JNK and p38 (Ichijo et al., 1997; Saitoh et al., 1998). In chick utricles, activation of JNK occurs upstream of cytochrome c release in hair cells exposed to aminoglycosides (Matsui et al., 2004). Our data in mammalian cells indicate that JNK signaling is upstream of caspase-9 activation. Thus it appears that aminoglycoside-induced hair cell death occurs via a mechanism that begins with the formation of ROS, resulting in activation of JNK. JNK activation lies upstream of release of cytochrome c from the mitochondria into the cytoplasm, which in turn is upstream of the activation of caspase-9. Finally, caspase-9 activity triggers the activation of downstream caspase-3 (Cunningham et al., 2002). These data add to our growing understanding of the molecular signaling pathways underlying aminoglycoside-induced hair cell death, and thus improve our chances of developing therapies to prevent or reverse it.

Acknowledgements

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