MINOCYCLINE PREVENTS GENTAMICIN-INDUCED OTOTOXICITY BY INHIBITING p38 MAP KINASE PHOSPHORYLATION AND CASPASE 3 ACTIVATION

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Abstract—Aminoglycosides are commonly used antibiotics that often induce ototoxicity leading to permanent hair cell loss and hearing impairment. We hereby examined whether minocycline protects hair cells from gentamicin-induced hair cell damage. Two millimolar gentamicin significantly induced outer hair cell damage and the addition of minocycline to gentamicin-treated explants significantly increased hair cell survival in a dose-dependent manner. Additionally, we demonstrated that gentamicin induced p38 MAPK phosphorylation, cytochrome c release, and caspase 3 activation in these cells and these remarkable changes were blocked by minocycline treatments. Furthermore, we showed that the inhibitor of p38 MAPK or the inhibitor of caspase 3 only partially blocked gentamicin-induced hair cell damage, and the pretreatment of explants with the inhibitor of p38 MAPK and the inhibitor of caspase 3 together exerted a synergic protective effect against gentamicin-induced hair cell damage. Our results suggest that minocycline blocks gentamicin-induced hair cell loss possibly by inhibition of three mechanisms: p38 MAPK phosphorylation, cytochrome c release, and caspase 3 activation. This finding may explain why minocycline has protective activity in a variety of apoptotic models. Therapeutic intervention by using minocycline or related drugs may be a novel means for preventing inner ear injury following the use of aminoglycoside. © 2005 Published by Elsevier Ltd on behalf of IBRO.

Key words: minocycline, apoptosis, caspase 3, p38 MAP kinase, ototoxicity, hair cell.

Significant hearing loss occurs in approximately 10% of the population. Decline in hearing can be caused by a variety of etiologies, including aging and therapeutic agents, such as aminoglycoside antibiotics (Arslan et al., 1999). The incidence of ototoxicity from aminoglycoside antibiotics is between 10 and 63% (Arslan et al., 1999). Recently, it has been suggested that aminoglycoside-induced hearing impairment may be caused by mutations in the mitochondrial DNA (Arslan et al., 1999). Since hair cells do not regenerate in the mammalian cochlea, their losses are irreversible and cumulative. Development of otoprotective drugs to protect these cells has been relatively elusive. Currently, there is no medical therapy to protect against hair cell loss, largely because the molecular mechanisms responsible for ototoxic- or noise-induced hair cell death remain undetermined. Recently, evidence has suggested that C-Jun N-terminal kinase (Pivola et al., 2000; Ulla et al., 2000; Wang et al., 2003), caspase 3 (Mangiardi et al., 2004; Liu et al., 1998), excitotoxicity (Darlington and Smith, 2003), as well as oxidative stress (Evans and Halliwell, 1999) are involved in ototoxic and noise-induced hair cell death.

Minocycline is a semisynthetic second-generation tetracycline which exerts anti-inflammatory effects that are completely separate and distinct from its antimicrobial actions (Ryan and Ashley, 1998). Clinical studies have suggested that minocycline and related tetracyclines have beneficial anti-inflammatory activities which may be useful for treating both rheumatoid arthritis as well as osteoarthritis (Ryan et al., 1996). Recently, the neuroprotective properties of minocycline in models of ischemic injury (global and focal ischemia; Yrjanheikki et al., 1998, 1999) have been shown to be due, in part, to indirect effects in inhibiting glial (astrocytic/microglial) caspase 1, iNOS activity, as well as p38 MAP kinase (p38 MAPK) phosphorylation (Yrjanheikki et al., 1998, 1999; Tikka et al., 2001). We have also demonstrated that minocycline is able to block 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced neurotoxicity by inhibiting neuronal p38 MAPK phosphorylation (Du et al., 2001). Additionally, Chen et al. (2000); Zhu et al. (2002) demonstrated that minocycline treatment delayed mortality or progression in a mouse model of Huntington disease and amyotrophic lateral sclerosis, presumably by inhibiting caspase 3 expression and cytochrome c release. They suggest that the primary target of minocycline is to block the release of cytochrome c. However, since considerable evidence suggests that phosphorylation of p38 MAPK regulates the release of cytochrome c (Zhuang et al., 2000; Cheng et al., 2001), minocycline may be able to block cytochrome c release by inhibition of p38 MAPK. Furthermore, since in almost all of these neuroprotective models, using specific inhibitors of these proteins alone provided much less neuroprotection than minocycline, we suggest that minocycline may target additional neuroprotective mechanisms than originally suggested. Most recently, it
Fig. 1. Administration of minocycline significantly blocks gentamicin-induced cell damage in cultured hair cells. (a, b) The number of outer hair cells/100 μm is decreased by 84±1.5% following gentamicin (2 mM; G) treatment for 24 h as compared with controls (C). Minocycline (10 μM) treatment blocks gentamicin-induced hair cell damage (G–M). (c) Minocycline blocks gentamicin-induced hair cell damage in a concentration-dependent manner. Photomicrographs are from a representative experiment. Values are expressed as number of surviving outer hair cells per 100 μm and % of control. Data represent the mean±S.E. values of triplicate determinations from a single but representative experiment repeated three times with similar results (*** P<0.001 by one-way ANOVA; scale bar=50 μm).
Fig. 2. Minocycline (10 μM; G–M) treatment inhibits gentamicin (G)-induced phosphorylation of p38 MAPK in hair cell layers. P-p38 MAPK was immunostained in representative hair cell explants 3 h after G treatment with or without treatment with minocycline (10 μM; left panel; see Experimental Procedures for details). Intact hair cells in explants were stained with phalloidin (right panel). The data are from a representative experiment repeated three times with similar results. Scale bar=50 μm.

Fig. 3. Minocycline (10 μM; G–M) treatment blocks gentamicin (G)-induced activated caspase 3. Active caspase 3 were immunostained in representative hair cell explants 16 h after G treatment with or without treatment with minocycline (left panel). Intact hair cells in explants were stained with phalloidin (right panel). The data are from a representative experiment repeated three times with similar results.
has been reported that Bcl-2 is induced by minocycline, accumulates in mitochondria, and may protect the organelles by antagonizing proapoptotic Bax, Bak, and Bid (Wang et al., 2004). Given the broad potential therapeutic efficacy of minocycline, understanding its neuroprotective mechanism(s) is of great importance.

EXPERIMENTAL PROCEDURES

Cochlear cultures

Cochlear explants were prepared from postnatal day 3 Sprague–Dawley rats by using the methods of Van De Water and Sobkowicz (Van de Water and Ruben, 1974; Sobkowicz et al., 1993; Pirvola et al., 2000). In short, after the bony-cartilaginous cochlear capsule was separated from the temporal bone, the intact organ of Corti was freed of all remaining bits of cartilage or bone and stripped off the stria vascularis from base to apex. The basal half of cochlea explants containing the basal and the middle turns was then dissected and cultured in Dulbecco’s modified Eagle medium (Invitrogen, Carlsbad, CA, USA) supplemented with 6 g/l of D-glucose, 1% N1-supplement (Sigma, St. Louis, MO, USA), and 100 units/ml of penicillin (Liu et al., 1998). On the second day, cultures were exposed for 3 h (p38), 16 h (caspase 3), or 24 h (cell toxicity) to 2 mM gentamicin (Invitrogen), a concentration that is in the range used by others (Dehne et al., 2002; Ding et al., 2002) with or without a 2-h minocycline (dissolved in dH2O) pretreatment. Explants were then fixed with 4% paraformaldehyde in PBS for 30 min and stained with a 1:100 dilution of fluorescein–phalloidin (Sigma) to identify intact auditory hair cells (Kuang et al., 1999).

All experiments conformed to Indiana University and international guidelines on the ethical use of animals. We also minimized the number of animals used and their suffering.

Assessment of hair cell survival

Numbers of inner hair cells and outer hair cells were quantified using a Nikon Diaphot-300 inverted fluorescence microscope with a 40× objective lens and an ocular grid. For each experimental point, three cochlea explants were assayed. In each cochlea, surviving inner hair cells and outer hair cells were counted from three randomly selected fields (100 μm length) in the middle segment of cultured cochlea explants which contained both basal and middle turns (Liu et al., 1998; Zheng and Gao, 1999). Each experiment was repeated at least three times.

Immunocytochemistry

After post-fixation, primary cultured hair cell explants were incubated overnight with antibodies against active caspase 3 (BD Biosciences) and phosphorylated p38 (Cell Signaling Technology) followed by visualization with the secondary goat biotinylated-conjugated polyclonal anti-mouse antibody (1:1000; Vector Laboratories, Burlingame, CA, USA) and 1:200 dilution of fluorescein–avidin (Vector Laboratories).

Protease activity (Du et al., 1997)

Treated explants were collected, washed three times with phosphate-buffered saline (PBS), pH 7.2, and resuspended in 100 μl precooled buffer (10 mM HEPES pH 7.4, 2 mM EDTA, 0.1% CHAPS, 5 mM DTT, 1 mM PMSF, 10 μg/ml aprotinin). Cells were allowed to swell on ice for 20 min. Following freeze
and thaw five times, lysates were centrifuged at 15,000 r.p.m. at 4 °C for 30 min and protein concentrations were determined (Pierce, Rockford, IL, USA). Extracts were either used immediately or stored at −80 °C. Aliquots of protein (30 μg) were incubated with 100 μM caspase 3 substrate (Ac-DEVD-pNA; Calbiochem, La Jolla, CA, USA) in a total volume of 1.0 ml at 37 °C. The colorimetric release of p-nitroaniline from the Ac–DEVD–pNA substrate was recorded every 10 min at 405 nm. Enzymatic activity for caspase 3 was linear over the range of protein concentrations used to calculate specific activity.

Cytochrome c

Immunoblot analysis was performed on cytoplasmic extracts from treated and untreated cultures. Cells were harvested in the 500 μl of ice-cold buffer A (50 mM Tris–HCl, pH 7.4; 1 mM EDTA; 1 mM DTT; complete protease inhibitor [Roche, Indianapolis, IN, USA]; 250 mM sucrose) after washing once with ice-cold PBS. The cells were disrupted by douncing 10 times with a pestle in a 7-ml Wheaton douncer. After centrifugation in a microcentrifuge at 1000×g for 10 min at 4 °C, the supernatants were further centrifuged at 12,000×g for 40 min. The resulting supernatants and pellets were used for immunoblot analysis. Aliquots (25 μg) of cytosol and pellets were size fractionated by SDS-PAGE electrophoresis (Nupage; Novex, San Diego, CA, USA) and transferred to a nitrocellulose membrane (Hybond; Amersham, Arlington Heights, IL, USA). The blots were probed with a monoclonal antibody against residues 93–104 of non-native cytochrome c (Pharmingen, San Diego, CA, USA) and visualized using enhanced chemiluminescence (Amersham).

**RESULTS**

To investigate the neuroprotective effects of minocycline on gentamicin-induced hair cell loss in vitro, we treated cultured neonatal rat cochlear explants with minocycline and gentamicin for 24 h. The explants were analyzed by immunohistochemistry to quantify phalloidin-stained hair cells. Gentamicin treatment significantly disrupted the normal pattern of phalloidin-stained outer hair cells. Gentamicin treatment significantly disrupted the normal pattern of phalloidin-stained outer hair cells and destroyed these cells (Fig. 1a). In explants exposed to 2 mM gentamicin for 24 h, 84±1.5% of outer hair cells were lost (Fig. 1b). In this model, we have found gentamicin significantly induces outer hair cell damage in both middle and basal turns. Inner hair cells in these regions show much less sensitivity to gentamicin toxicity (inner hair cells: 19% vs. outer hair cells: 84%). Explants that received treatments of minocycline and gentamicin showed increased phalloidin-stained outer hair cells, ranging from 16±1.5% of control following minocycline treatment (Fig. 1c). The neuroprotective effect of minocycline was dose-dependent (IC50 approximately 1 μM; Fig. 1c). Minocycline alone did not alter the number of phalloidin-stained hair cells significantly (data not shown).

Since caspase 3 and p38 MAPK have recently been proposed to mediate (at least in part) neuronal death and minocycline-induced neuroprotection (Chen et al., 2000; Du et al., 2001; Tikka et al., 2001), we measured both

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Fig. 5. ZVAD (100 μM) alone and together with SB203580 (SB, 10 μM) significantly blocks gentamicin-induced cell toxicity. Data represent the mean±S.E. values of triplicate determinations from a single but representative experiment repeated three times with similar results (** P<0.01, *** P<0.001 by one-way ANOVA).
phosphorylated p38 MAPK (p-p38; Fig. 2) and active caspase 3 (Fig. 3) in hair cells treated with gentamicin. At 3 and 16 h following gentamicin treatments, p-p38 and active caspase 3 were significantly upregulated in outer hair cells as determined by immunocytochemistry, respectively. Moreover, these changes were markedly blocked in the presence of minocycline (Fig. 3). Furthermore, to examine if gentamicin-induced phosphorylation of p38 MAPK and caspase 3 activation contribute to gentamicin toxicity, we pretreated explants with SB 203580 (10 μM), an inhibitor of p38 MAPK, and DEVD (200 μM), an inhibitor of caspase 3, alone and together for 2 h, followed by exposure to gentamicin (2 mM). Surprisingly, SB 203580 and DEVD each alone were apparently only able to rescue 50% or fewer cells, far less than minocycline’s effects (Fig. 4). However, treating explants with these two inhibitors together showed synergetic protective effects against gentamicin’s toxicity similar to the effects of minocycline exposure (Fig. 4). These results suggest that a p38 MAPK-independent caspase 3 pathway contributes to gentamicin-induced hair cell toxicity, and blocking both p38 MAPK and caspase 3 pathways together has a synergetic protective effect. Minocycline-induced neuroprotection is possibly mediated by inhibition of these two pathways. To further confirm our hypothesis, we tested ZVAD, a pan caspase inhibitor in this model, and found that ZVAD had a similar effect to DEVD (Fig. 5). In the mechanistic study, we found that inhibition of an early event, phosphorylation of p38 MAPK by using its inhibitor, SB 203580, significantly blocked cytochrome c release, which was consistent with previous reports using other models (Zhuang et al., 2000; Cheng et al., 2001; Fig. 6). Since we found that SB 203580 only partially inhibited gentamicin-induced caspase 3 activity (Fig. 7), we suggest that SB 203580 may specifically inhibit cytochrome c-mediated caspase 3 activation. Additional protective effect by DEVD may result from inhibition of cytochrome c independent caspase 3 activation (Iordanov et al., 2000).

**DISCUSSION**

Hair cell damage which is associated with hearing loss can be caused by the aminoglycoside antibiotics. Unfortunately, their losses are irreversible and cumulative in the mammalian cochlea. Development of otoprotective drugs to protect these cells has therefore been relatively elusive. Currently, there is no medical therapy to protect against hair cell loss, and the molecular mechanisms responsible for ototoxic- or noise-induced hair cell death until now have been largely unknown.

In this study, we found gentamicin significantly induced outer hair cell damage in both middle and basal turns.
Additionally, inner hair cells showed much less sensitivity to gentamicin toxicity. Furthermore, minocycline exposure significantly prevented gentamicin-induced outer hair cell damage. To our knowledge, this is the first demonstration that gentamicin-induced hair cell destruction can be significantly blocked by minocycline at relatively low concentrations (IC50 approximately 1 M). This finding, coupled with our and other recent reports demonstrating the neuroprotective effects of minocycline in a variety of neuronal disorder models (Yrjanheikki et al., 1998; Chen et al., 2000; Du et al., 2001; Zhu et al., 2002; Popovic et al., 2002; Wells et al., 2003), supports the conclusion that minocycline may be able to inhibit cell death induced by a variety of toxins.

Since it had been shown that minocycline was able to block cytochrome c release from mitochondria directly (Zhu et al., 2002) or indirectly by induction of Bel-2 (Wang et al., 2004), and it has been well documented that cytochrome c release and caspase 3 activation in gentamicin-induced hair cell death (Mangiardi et al., 2004), we investigated whether gentamicin-induced cytochrome c release in hair cells could be blocked by minocycline. As expected, minocycline significantly blocked cytochrome c release. Additionally, in previous studies, it had been demonstrated that minocycline exerted its neuroprotective effects by inhibiting p38 MAPK phosphorylation and caspase 3 activation (Chen et al., 2000; Du et al., 2001; Tikka et al., 2001). In this study, we demonstrated that minocycline was able to block p38 MAPK phosphorylation and caspase 3 activation in hair cells. However, since SB203580 is only able to partially inhibit caspase 3 activity, a p38 MAPK-independent caspase 3 pathway may exist. Furthermore, since SB 203580 and DEVD each alone were apparently only able to rescue 50% or fewer cells, far less than minocycline’s effects (Fig. 4), and p38 inhibitor only blocked approximately 50% of the caspase 3 activation that minocycline did, our results suggest that in addition to blocking the p38 MAPK-dependent caspase 3 and the p38 MAPK-independent caspase 3 pathways, minocycline must block cell death by inhibiting p38 MAPK-induced cell death which is independent of cytochrome c release and caspase 3 activation. In other words, minocycline has much broader effects than just p38 MAPK inhibition or just caspase 3 inhibition. Our data suggest that both hyperphosphorylation of p38 MAPK and activation of caspase 3 play important roles in gentamicin-induced ototoxicity. Furthermore, since inhibitors of p38 MAPK and caspase 3 together exert a similar protective effect to minocycline and since minocycline blocks both p38 MAPK phosphorylation and caspase 3 activation, we suggest that inhibition of p38 MAPK and cytochrome c-mediated caspase 3 pathway may mediate minocycline’s protective effects against gentamicin toxicity. Interestingly, changes of p-p38 MAPK and active caspase 3 seem to mainly occur in outer hair cells, which is consistent with toxicity results. We caution, however, that minocycline reduces p38 MAPK phosphorylation presumably by inhibiting an “upstream” kinase (Du et al., 2001).
Additional work will be required to delineate minocycline’s exact cellular target(s) in the p38 MAPK pathway. Recently, inflammation has been reported to be widely involved in neurodegeneration. It has been reported that inflammation and white blood cells may be involved in hair cell damage (Huang et al., 1990; Bodmer et al., 2002). Additionally, nitric oxide (NO) has been detected in middle ear effusion and exposure to NO has caused irreversible morphologic changes in isolated outer hair cells, suggesting NO may be involved in the development of sensorineural hearing loss (Jung et al., 2003). Furthermore, proteins of cytokines including interleukin-1 and tumor necrosis factor α were found in type I fibrocytes and root cells located within the spiral ligament (Adams, 2002). Since minocycline exerts its neuroprotective effects by inhibiting glial activation and the subsequent release of NO and perhaps cytokines, such as IL-1β (Yrjanheikki et al., 1998, 1999; Chen et al., 2000; Du et al., 2001; Tikka et al., 2001), it is very likely that such “anti-inflammatory” actions of minocycline may contribute to the protective properties we observe in gentamicin-induced hair cell damage. Further investigation will therefore be required to investigate the exact role(s) of inflammation in effects of gentamicin and minocycline on hair cells.

Since minocycline has a robust protective activity in gentamicin-induced hair cell damage, chemically modified tetracyclines, like minocycline, may prove effective in preventing and (or) altering the progression of human deafness associated with aminoglycoside use as well as other etiologies associated with hair cell damage. Furthermore, given that both p38 MAPK phosphorylation and release of cytochrome c/caspase 3 activation play important roles in many neurological disorders, inhibition of these pathways by minocycline explains, at least partially, the broad spectrum of this drug to inhibit cell death in many in vivo and in vitro models.

REFERENCES


