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ROLE OF INHIBITOR OF APOPTOSIS PROTEIN IN GENTAMICIN-INDUCED COCHLEAR HAIR CELL DAMAGE

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Abstract—Apoptotic cell death is considered to play a key role in gentamicin-induced cochlear hair cell loss. Inhibitor of apoptosis proteins (IAPs) are important regulators of apoptosis that can prevent activation of effector caspases. This study was designed to investigate the possible involvement of X-linked inhibitor of apoptosis protein (XIAP) in hair cell death due to gentamicin. Basal turn organ of Corti explants from postnatal day (p) p3 or p4 rats were maintained in tissue culture and were exposed to 35 μM gentamicin for up to 48 h. Effects of specific XIAP inhibitors on gentamicin-induced hair cell loss and caspase-3 activation were examined. XIAP inhibitors increased gentamicin-induced hair cell loss but an inactive analog had no effect. Caspase-3 activation was primarily observed at 36 or 48 h in gentamicin-treated hair cells, whereas caspase-3 activation peaked at 24–36 h when explants were treated with gentamicin and an XIAP inhibitor. The inhibitors alone had no effect on hair cells. Finally, a caspase-3 inhibitor decreased caspase-3 activation and hair cell loss induced by gentamicin and an XIAP inhibitor, but caspase-8 and -9 inhibitors did not. The results indicate that XIAP normally acts to decrease caspase-3 activation and hair cell loss during gentamicin ototoxicity, as part of a protective response to potentially damaging stimuli. © 2007 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: X-linked inhibitor of apoptosis protein, XIAP, organ of Corti, caspase, programmed cell death, ototoxicity.

Apoptosis is a pathophysiological process that plays critical roles in cell death and tissue homeostasis (Zimmermann et al., 2001). Apoptosis mainly occurs through the sequential actions of caspases. Two upstream initiator caspases, caspase-8 and caspase-9, and their associated extrinsic and intrinsic pathways are well known. The extrinsic pathway begins with activation of cell surface death receptors. Activation of death receptors cleaves caspase-8, which in turn activates downstream effector caspases such as caspase-3. In contrast, the intrinsic pathway is initiated by changes in mitochondrial membrane permeability. Cytochrome c released from mitochondria forms a protein complex with Apaf 1. This in turn activates caspase-9 and the downstream effector caspases. Activated effector caspases then cleave critical intracellular proteins to induce the final stages of cell death.

Gentamicin is a widely used antibiotic. However, its clinical usage is sometimes limited by the side effects of nephrotoxicity and ototoxicity. Broad-spectrum caspase inhibitors decrease hair cell death induced by aminoglycoside, suggesting that some hair cell death after aminoglycoside exposure is mediated by the action of caspases and apoptosis (Forge and Li, 2000; Matsui et al., 2002). The mechanisms of aminoglycoside-induced hair cell apoptosis are not fully known. However, it has been reported that caspase-9 is the primary upstream caspase mediating neomycin-induced caspase-3 activation in vestibular hair cells because caspase-9 inhibition but not caspase-8 inhibition reduced caspase-3 activation and cell death of vestibular hair cells induced by neomycin (Cunningham et al., 2002).

The activation of caspases is tightly regulated to avoid inappropriate cell death. Recent studies have revealed that caspases are regulated at several checkpoints in the apoptotic pathway. The inhibitor of apoptosis proteins (IAPs) represent a family of endogenous caspase inhibitors that share a conserved structure known as the BIR (baculovirus inhibitory repeat) domain, a zinc-binding region consisting of less than 70 amino acid (Deveraux et al., 2001; Schimmer, 2004a). X-linked inhibitor of apoptosis protein (XIAP) is the best characterized of the IAP family members in terms of the mechanism of caspase inhibition. XIAPs have three BIR domains. Their BIR2 and BIR3 domains are responsible for inhibiting caspases-3 and caspase-9, respectively (Takahashi et al., 1998; Deveraux et al., 1997).

The role of IAPs described above suggests that IAPs could function as negative regulators of gentamicin-induced apoptosis of hair cells. Moreover, Chan et al. (2007) and Cooper et al. (2006) have recently shown that inducing expression of XIAP in hair cells protects them against cisplatin ototoxicity. However, it is not clear whether IAPs function normally in the HC to limit cell death. To further understand the molecular mechanisms of hair cell death, we examined the effects of XIAP inhibitors on gentamicin-induced auditory hair cell loss.

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Abbreviations: BIR, baculovirus inhibitory repeat; IAP, inhibitor of apoptosis proteins; p, postnatal day; PBS, phosphate-buffered saline; XIAP, X-linked inhibitor of apoptosis protein.

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EXPERIMENTAL PROCEDURES

Culture techniques

The basal turn of the organ of Corti was dissected from Sprague–Dawley rats at postnatal day 3 (p3) or 4 (p4) and cultured based on the methods of Van de Water and Ruben (1974) and Sobkowicz et al. (1993). The local animal subjects committee of the San Diego VA Healthcare System approved the surgical procedures in accordance with the guidelines laid down by the National Institutes of Health regarding the care and use of animals for experimental procedures. All efforts were made to minimize the number of animals used and their suffering. Explants were maintained in Dulbecco's modified eagles medium (DMEM) with 10% fetal bovine serum (FBS), 25 mM Hepes and 30 U/ml penicillin and were cultured in an incubator at 37 °C with 5% CO_2 and 95% humidity. Gentamicin alone cultures were maintained in the above-described initial medium for 16–24 h and then exposed to medium containing 35 μM gentamicin for up to 48 h. In studies for XIAP inhibitors and/or caspase inhibitors, cultures were pretreated with the culture medium containing the test substances for 16 h to allow the substances to penetrate hair cell membranes. The cultures were then exposed to medium containing 35 μM gentamicin and the test substances for up to 48 h. At least six cultures were used for each condition tested.

Cytochemistry

At the end of culture, the explants were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min and then permeabilized with 5% Triton X-100 (Sigma, St. Louis, MO, USA) in PBS with 10% fetal bovine serum for 10 min. The specimens were stained with a conjugated phalloidin–Texas Red probe (1:100, Texas Red X–phalloidin, Invitrogen, Carlsbad, CA, USA) at room temperature for 1 h. Phalloidin is a specific marker for cellular F-actin and labels stereociliary arrays and cuticular plates of hair cells.

Caspase-3 activation was detected using fam-DEVD-fmk (Chemicon, Temecula, CA, USA), a fluorescent caspase-3 substrate, using the manufacturer's protocol. After explants were cultured for 12, 24, 36 or 48 h in the culture media containing 35 μM gentamicin alone or 35 μM gentamicin plus 20 μM compound 1396-34, they were cultured for one more hour in the culture media of the same composition in which the fluorescent substrate was added (Cunningham et al., 2002; Zhang et al., 2003). At the end of the culture, the explants were fixed with 4% paraformaldehyde in PBS, permeabilized with 5% Triton and then

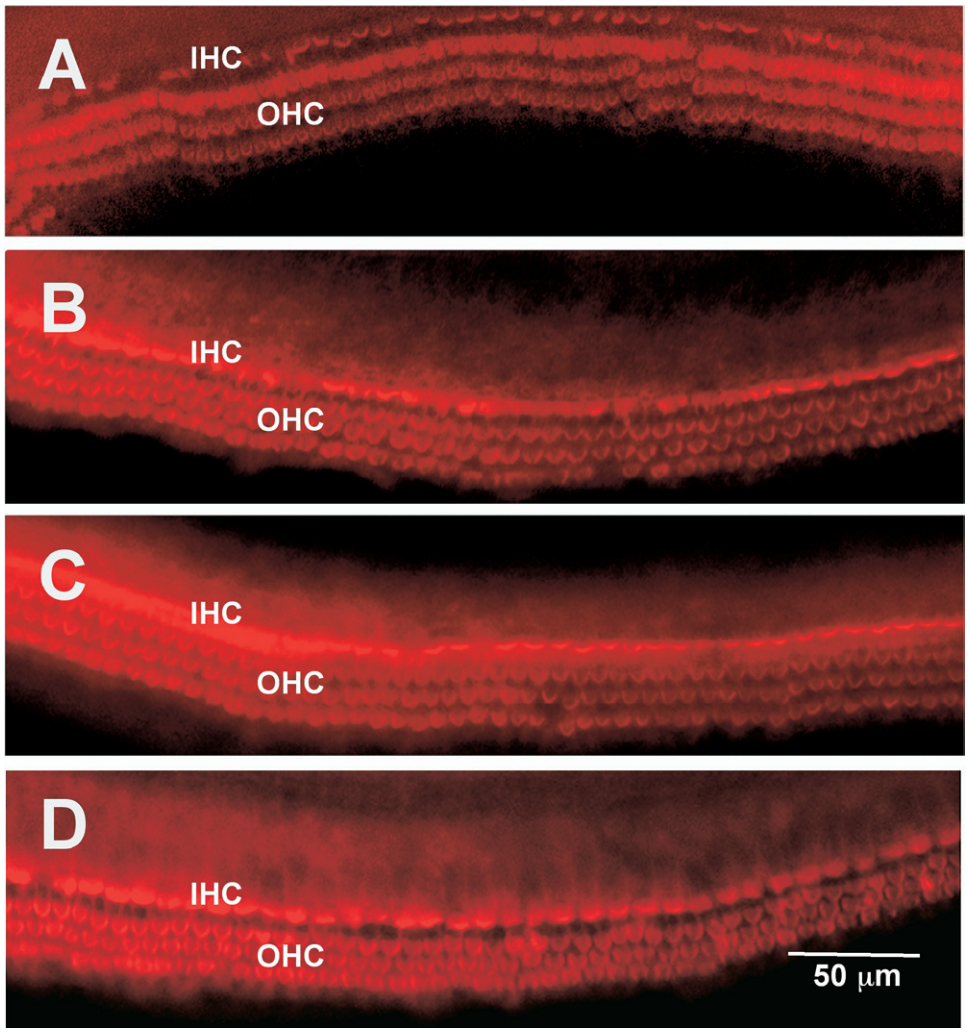

Fig. 1. Representative fluorescence micrographs of Texas Red phalloidin-labeled hair cells treated with XIAP inhibitors alone for 48 h. (A) control. (B) compound 1396-11, 20 μM. (C) Compound 1396-34, 20 μM. (D) Compound 1396-28, 20 μM. Neither the inhibitors (1396-11, 1396-34) nor the inactive control compound (1396-28) affected inner hair cell (IHC) or outer hair cell (OHC) morphology.