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Galangin prevents aminoglycoside-induced ototoxicity by decreasing mitochondrial production of reactive oxygen species in mouse cochlear cultures

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HIGHLIGHTS

- Amikacin results in ototoxicity due to the overproduction of reactive oxygen species.
- Galangin prevents amikacin-provoked increase in ROS production in cochlear explants.
- Galangin reduced apoptotic cell death of hair cells in amikacin-treated cochlear explants.

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ABSTRACT

Amikacin is a semi-synthetic aminoglycoside widely used to treat infections caused by gentamicin-resistant gram-negative organisms and nontuberculous mycobacteria. However, the use of this agent often results in ototoxicity due to the overproduction of reactive oxygen species (ROS). Galangin, a natural flavonoid, has been shown to play a protective role against mitochondrial dysfunction by reducing mitochondrial ROS production.

In this study, the effect of galangin on amikacin-induced ototoxicity was examined using cultures of cochlear explants. Immunofluorescent staining showed that treatment of inner hair cells (IHCs) and outer hair cells (OHCs) with galangin significantly decreased damage induced by amikacin. Moreover, pretreatment with galangin resulted in decreased amikacin-provoked increase in ROS production in both types of hair cells by MitoSOX-red staining. Attenuation of apoptotic cell death was assessed immunohistochemically using active caspase-3 antibody and with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, compared to explants exposed to amikacin alone ($P < 0.05$). These results indicate that galangin protects hair cells in the organ of Corti from amikacin-induced toxicity by reducing the production of mitochondrial ROS.

The results of this study suggest that galangin can potentially be used as an antioxidant and antiapoptotic agent to prevent hearing loss caused by aminoglycoside induced-oxidative stress.

1. Introduction

Aminoglycosides (AGs) are widely used to treat gram-negative bacterial infections, especially aerobic gram-negative bacteria due to their low cost, documented efficacy, and rare incidence of allergic reactions (Rybak and Ramkumar, 2007). Side effects, such as ototoxicity (Greenwood, 1959; Hock and Anderson, 1995) and nephrotoxicity, often associated with this drug class have caused

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decline in clinical use. However, AGs are still used to treat life-threatening infections due to their broad spectrum of antimicrobial activity and low rates of drug resistance. For example, AGs are considered as the first-line therapy for severe, life-threatening neonatal sepsis, and the World Health Organization recommends their use for the treatment of drug-resistant tuberculosis (Falzon et al., 2011; Mingeot-Leclercq et al., 1999). The incidence of ototoxicity in patients treated with AGs is nearly 25% (Fee, 1980). The primary manifestations of AG-induced ototoxicity include hearing loss and dizziness. Since these symptoms cannot be detected during the initial treatment, diagnoses are often delayed and difficult to attribute to a specific cause. The incidence of AG-induced ototoxicity can be further underestimated due to its disproportionate effect on high frequency hearing; some patients do not realize that they are suffering from hearing loss until it extends to whole frequencies. It has been estimated that nearly 50% of patients with a history of AG treatment have experienced appreciable hearing loss (Fausti et al., 1992). The semi-synthetic AG, amikacin was developed to treat infections caused by gentamicin-resistant gram-negative organisms and/or nontuberculous mycobacteria (Huth et al., 2011). Amikacin has been known to promote cochlear toxicity more frequently than vestibular damage (Matz, 1993).

Reactive oxygen species (ROS) physiologically produced in mitochondria are involved in signaling pathways that mediate adaptive responses to stress and also help regulate cellular growth and differentiation (Finkel and Holbrook, 2000). However, overproduction can result in ROS concentrations that alter enzymatic and ion channel activities, and even induce apoptosis (Cheng et al., 2005). Multiple studies have linked ROS to AG-induced ototoxicity (Priuska and Schacht, 1995; Sha and Schacht, 1999).

Galangin (3,5,7-trihydroxyflavone) is a naturally-occurring flavonoid antioxidant found in lesser galangal (*Alpinia officinarum*, Hance) at a high concentration (Chien et al., 2015). Galangin has been shown to exhibit antimutagenic, anticlastogenic, antioxidant, radical scavenging, metabolic enzyme modulating, bactericidal, and antifibrotic properties (Pepeljnjak and Kosalec, 2004; Wang et al., 2013). Further, galangin was found to inhibit caspase-dependent mitochondrial cell death by decreasing ROS production in a mouse model of ischemic stroke (Li et al., 2012). Although studies have demonstrated that galangin exerts protective effects against oxidative stress-mediated cell death, its role in the prevention/treatment of inner ear disease is unknown.

In this study, we investigated the use of galangin as a protective agent against amikacin-induced ototoxicity by evaluating its effect on mitochondrial ROS production and apoptosis in mouse cochlear cultures.

2. Materials and methods

2.1. Culture of mouse cochlear explants

Primary cochlear explants were prepared from postnatal day (P) 3 Institute for Cancer Research (ICR) mice were purchased from Hyochang Science (Daegu, Republic of Korea). The dissected organs of Corti were attached to the four-well culture dishes and subsequently incubated with culture medium composed of high-glucose Dulbecco's Modified Eagle's Medium (DMEM; Hyclone, Logan, UT, USA) containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA) and ampicillin (10 μg/mL; Life Technologies, Carlsbad, CA, USA) in a humidified atmosphere of 5% CO_2 at 37 °C. The cultured organs of Corti were divided into six groups for galangin treatment: control (CT, $n = 4$), amikacin alone (AK, $n = 4$), amikacin with pretreated decyl triphenylphosphonium (AK + dTPP, $n = 4$), amikacin with pretreated MitoQ10 (AK + MitoQ10, $n = 4$), amikacin with pretreated galangin (AK + GG, $n = 4$), and galangin alone (GG, $n = 4$). After 16 h incubation, organs of Corti were treated with galangin (10 μM in DMSO; Sigma, St. Louis, MO, USA) or MitoQ10 (500 nM in DMSO) or dTPP (500 nM in DMSO) diluted in culture medium for 1 h in a humidified atmosphere of 5% CO_2 at 37 °C. After 1 h incubation, amikacin (1 mM; Ildong Pharmaceutical Co., Daegu, Republic of Korea) was added to the AK, AK + GG, AK + MitoQ10, and AK + dTPP groups.

All animal experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of Kyungpook National University and approved by the Committee on the Ethics of Animal Experiments of the Kyungpook National University.

2.2. Histological evaluation

To investigate the protective effects of galangin on amikacin-induced ototoxicity in the organ of Corti, we examined the morphology of inner hair cells (IHCs) and outer hair cells (OHCs) within the organs of Corti. At the end of the 48 h incubation periods, all cochlear explants were washed with phosphate buffered saline (PBS), fixed with 4% paraformaldehyde (PFA, pH 7.4) in PBS for 15 min, and permeabilized with 0.1% Triton X-100 in PBS (PBS-Tx) for 30 min at room temperature (RT). Permeabilized samples were blocked with 5% normal goat serum diluted in PBS-Tx for 1 h at RT and then stained with Alexa Fluor® 488 or 555-conjugated phalloidin (1:1,000; Invitrogen-Molecular Probes, Eugene, OR, USA) in PBS-Tx for 3 h at RT. The specimens were rinsed three times with PBS and mounted on glass slides using Fluoromount (Sigma–Aldrich, St. Louis, MO, USA). Images were captured using a Zeiss Axio Imager A2 fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

2.3. Determination of mitochondrial ROS levels

MitoSOX-red (Invitrogen, Carlsbad, CA, USA) is a novel fluorogenic indicator of superoxide generated specifically from mitochondria (Robinson et al., 2006). At the end of the 30 h incubation periods, all cochlear explants were washed with PBS and stained with MitoSOX-red (5 μM) for 10 min in a humidified atmosphere of 5% CO_2 at 37 °C. After washing with PBS, the specimens were visualized using a Zeiss Axio Imager A2 fluorescence microscope.

2.4. Determination of mitochondrial membrane potential (ΔY_m)

Mitochondrial membrane potential was estimated using the cationic fluorescent dye, MitoProbe™ JC-1 (Molecular Probes, Eugene, OR, USA) according to the manufacturer's instructions. At the end of the 30 h incubation periods, all cochlear explants were washed with PBS and incubated with JC-1 (2 μM) for 50 min in 5% CO_2 at 37 °C in the dark. To confirm the sensitivity of JC-1, the cochlear explants were not treated with any drugs were added 50 μM carbonyl cyanide 3-chlorophenylhydrazone (CCCP) before treatment of JC-1 and incubated for 5 min at 37 °C. We quantified red over green JC-1 fluorescence ratios by splitting the images to bright field and measuring the average grayscale using Image J software (http://imagej.nih.gov/ij/).

2.5. Immunohistochemical analysis and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

To assess apoptotic cell death in the organs of Corti, we conducted immunohistochemistry using anti-active caspase-3 and also evaluated DNA fragmentation using the TUNEL assay. At the end of the 48 h incubation periods, all cochlear explants were fixed,