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Erdosteine protects HEI-OC1 auditory cells from cisplatin toxicity through suppression of inflammatory cytokines and induction of Nrf2 target proteins

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ABSTRACT

Cisplatin has many adverse effects, which are a major limitation to its use, including ototoxicity, neurotoxicity, and nephrotoxicity. This study aims to elucidate the protective mechanisms of erdosteine against cisplatin in HEI-OC1 cells. Pretreatment with erdosteine protects HEI-OC1 cells from cisplatin-medicated apoptosis, which is characterized by increase in nuclear fragmentation, DNA laddering, sub-G_0/G_1 phase, H2AX phosphorylation, PARP cleavage, and caspase-3 activity. Erdosteine significantly suppressed the production of reactive nitrogen/oxygen species and pro-inflammatory cytokines such as tumor necrosis factor-α, interleukin (IL)-1β, and IL-6 in cisplatin-treated cells. Studies using pharmacologic inhibitors demonstrated that phosphatidylinositol-3-kinases (PI3K) and protein kinase B (Akt) have protective roles in the action of erdosteine against cisplatin in HEI-OC1 cells. In addition, pretreatment with erdosteine clearly suppressed the phosphorylation of p53 (Ser15) and expression of p53-upregulated modulator of apoptosis. Erdosteine markedly induces expression of NF-E2-related factor 2 (Nrf2), which may contribute to the increase in expression of glutathione redox genes γ-L-glutamate-L-cysteine-ligase catalytic and γ-L-glutamate-L-cysteine-ligase modifier subunits, as well as in the antioxidant genes HO-1 and SOD2 in cisplatin-treated HEI-OC1 cells. Furthermore, the increase in expression of phosphorylated p53 induced by cisplatin is markedly attenuated by pretreatment with erdosteine in the mitochondrial fraction. This increased expression may inhibit the cytosolic expression of the apoptosis-inducing factor, cytochrome c, and Bax/Bcl-xL ratio. Thus, our results suggest that treatment with erdosteine is significantly attenuated cisplatin-induced damage through the activation of Nrf2-dependent antioxidant genes, inhibition of pro-inflammatory cytokines, activation of the PI3K/Akt signaling, and mitochondrial-related inhibition of pro-apoptotic protein expression in HEI-OC1 auditory cells.

1. Introduction

Cisplatin is a chemotherapeutic agent widely used in the treatment of solid tumors (Kelland, 2007), but its use has been limited by a variety of adverse effects such as ototoxicity (Rybak et al., 2009). Several previous studies have described the effect of cisplatin on the inner ear. Cisplatin-mediated damage to inner-ear structures begins from the basal turn and progresses to the apical turn of the cochlea (Moroso and Blair, 1983; Rybak et al., 2007) and cisplatin damages the spiral ganglia and stria vascularis in the inner ear (Tange and Vuzevski, 1984). The major mechanism of the anticancer activity of cisplatin involves the generation of nuclear DNA adducts that block tumor cell proliferation and induce apoptosis (Jordan and Carmo-Fonseca, 2000). Accumulating evidence has shown that ROS have been implicated as the main mediators of apoptosis caused by cisplatin in the inner ear and cochlear structures (Clerici et al., 1996; Rybak et al., 2007).

Administration of cisplatin adversely affects the outer hair cells (OHC) by causing intracellular depletion of the endogenous antioxidant, glutathione (Ravi et al., 1995; Rybak et al., 1999, 2000) and also decreases the activity of the enzymes that regenerate glutathione and antioxidant enzymes in the inner ear such as glutathione peroxidase, glutathione reductase, superoxide dismutase (SOD), and catalase (Ravi et al., 1995; Rybak et al., 1999, 2000). As a result, ROS levels increase

Abbreviations: AIF, apoptosis-inducing factor; Cisplatin, *cis*-diammine-dichloroplatinum II; Nrf2, NF-E2-related factor 2; OC, organ of Corti; PARP, poly ADP ribose polymerase; PUMA, p53-upregulated modulator of apoptosis.

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in the cochlear tissue, which generates a toxic environment for the cells. Several investigations with antioxidative reagents have already been conducted in animal models with a varying degree of success. The reagents investigated include ebselen, flunarizine, glutathione, lactate, L-methionine, N-acetyl-cysteine (NAC), sodium thiosulfate, and other agents (Kaltenbach et al., 1997; Kim et al., 2009; Korver et al., 2002; Nader et al., 2010; Saliba et al., 2010; So et al., 2006).

Erdosteine is a thiol derivative containing two sulfhydryl groups (−SH) that act as free radical scavengers and antioxidants, following their release during hepatic metabolism (Braga et al., 2010; Dechant and Noble, 1996). Several studies recently have demonstrated the free radical scavenging effects of erdosteine metabolites for various drug-induced conditions including cardiotoxicity, hepatotoxicity, nephrotoxicity, and ischemia–reperfusion injury (Erarslan et al., 2011; Fadillioglu et al., 2003; Lee et al., 2010; Ozyurt et al., 2004; Selcoki et al., 2007). Furthermore, oral treatment with erdosteine, attenuated the increase in serum creatinine level following an intraperitoneal injection of cisplatin (Yilmaz et al., 2004), and prevented cisplatin-induced in vivo nephrotoxicity and hepatic oxidative injury in rats (Koc et al., 2005; Ozyurt et al., 2004). In addition, there is in vivo evidence of the protective effect of erdosteine against cisplatin in rats (Kalcioglu et al., 2005) and guinea pigs (Waissbluth et al., 2012). In contrast, the intratympanic injection of erdosteine significantly increased the threshold of distortion product otoacoustic emission (DPOAE) and the complete destruction of the stereocilia of OHC and it also induced diffuse inflammatory reactions and otitis of the middle ear in cisplatin-treated guinea pigs (Saliba and El Fata, 2012). Although the protective and deleterious effects of erdosteine have been reported, the exact mechanisms of its modulation of cisplatin-mediated ototoxicity have not been elucidated in experimental models.

PI3Ks are a family of enzymes involved in cellular functions such as cell growth, proliferation, differentiation, motility, survival, and intracellular trafficking. PI3K is activated by G protein–coupled receptors and tyrosine kinase receptors (Leevers et al., 1999; Ullrich and Schlessinger, 1990) and activates PKB/Akt, which mediates cell survival through the regulation of numerous proteins such as glycogen synthase kinase-3β (GSK-3β), Bcl-2-associated death promoter (Bad), NF-*k*B, and Bcl-xL (Crowder and Freeman, 2000; Graff et al., 2000; Jones et al., 2000). Therefore, PI3K activation with subsequent elevation in the levels of phosphorylated PKB/Akt, can protect cells from apoptosis induced by cytotoxic drugs and contribute to drug resistance. Considerable evidence shows that the PI3K/Akt pathway plays a role in the regulation of Nrf2 activation, subsequent Nrf2 nuclear translocation, and induction of antioxidant response element (ARE)-mediated phase II genes such as HO-1 (Hwang and Jeong, 2010; Zhang et al., 2012). The Nrf2-Keap1 pathway is likely the central antioxidant signaling mechanism. Activated Nrf2 binds to ARE and activates the transcription of many cytoprotective genes that encode detoxifying enzymes and antioxidant proteins. It also confers resistance against xenobiotic drugs and oxidative stresses. Recently, a study showed that phosphorylation and activation of Akt are involved in the expression of Nrf2 and HO-1 (Nguyen et al., 2013). The major purpose of the current study was to examine the role of erdosteine in the protection of HEI-OC1 cells against cisplatin-induced toxicity.

2. Methods and materials

2.1. Chemicals and reagents. Cell culture supplies were purchased from Gibco by Life Technologies, Inc. (Life Technologies, Grand Island, NY, USA). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Wizard Genomic DNA purification kit was purchased from Promega (Madison, USA). Antibodies for PARP, p53, AIF, Bax, Bcl-xL, caspase-3, cytochrome-c, Nrf2, PUMA, SOD1, PI3K p85, Tom20, and p-PI3K p85 (Y508) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, USA). Antibodies against phospho-p53 (Ser15), p-Akt (Ser473), Akt, p-ERK 1/2 (T202/Y204), p-H2AX (Ser139) and ERK1/2 were purchased from Cell Signaling Technology Inc. (USA). Activated caspase-3 antibody was obtained from Abcam (UK). West save gold reagents for the immunoreactive signal detection were purchased from Abfrontier (Young In Frontier, Korea) and the secondary antibodies including anti-rabbit, anti-goat, and anti-mouse Ig conjugated with horseradish peroxidase (HRP) were purchased from Bethyl Laboratories, Inc. (USA).

2.2. Cell culture and MTT assay. HEI-OC1 is a conditionally immortalized auditory cell line, which was established, defined, and characterized by Kalinec et al. (Kalinec et al., 2003). HEI-OC1 cells (5×10^4 cells per well) were plated in a 24-well plate and incubated overnight. Cells were treated with varying doses of cisplatin for 30 h with or without pretreatment with 2 mM erdosteine for 3 h. To determine the cell viability, MTT (0.5 mg) was added to 1 mL of cell suspension for 4 h. The cells were washed three times with phosphate-buffered saline (PBS, pH 7.4), and the insoluble formazan product was dissolved in dimethyl sulfoxide (DMSO). The optical density (OD) of each culture well was measured using a microplate reader (Molecular Devices Co., Sunnyvale, CA, USA) at 590 nm. The OD of the control cells was denoted as 100% viability.

2.3. 4′-6-Diamidino-2-phenylindole (DAPI) staining of nuclei. The nuclei of HEI-OC1 cells were stained with the chromatin dye, 4′-6-diamidino-2-phenylindole (DAPI). Cells were fixed with 4% paraformaldehyde for 10 min at room temperature (RT), washed twice with PBS, and then incubated with 10 μM DAPI in PBS at RT for 30 min. After three washes, cells were observed under a fluorescence microscope (IX71, Olympus, Japan).

2.4. DNA fragmentation assay. Genomic DNA was isolated from cultured cells with the Wizard Genomic DNA purification kit (Promega). Briefly, the medium was removed, and the cells were lysed with lysis buffer followed by 1 h incubation with RNase A. Cell lysate was precipitated for proteins and spun at 13,000 rpm for 20 min. The supernatant was precipitated with isopropanol for isolation of DNA. After sequential washes with 70% and 100% alcohol, DNA was hydrated and quantified. Equal amounts (10 μg) of DNA were separated on a 1.5% agarose gel containing ethidium bromide. The gel was then photographed under ultraviolet (UV) luminescence.

2.5. Measurement of proinflammatory cytokines by enzyme-linked immunosorbent assay (ELISA). The extracellular secretion of proinflammatory cytokines in cisplatin-treated HEI-OC1 cells with or without erdosteine for 24 h was measured in culture supernatants. The levels of secreted proinflammatory cytokines including TNF-α, IL-1β, and IL-6 were determined by ELISA (Quantikine Cytokine Kits, R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. The minimum detectable concentrations of TNF-α, IL-1β, and IL-6 were 1.6, 1.0, and 0.7 pg/mL, respectively. A standard curve was obtained for each assay plate using the respective serially diluted recombinant proteins.

2.6. Semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) and quantitative real-time (q)-PCR. Following the extraction of total RNA from HEI-OC1 auditory cells using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol, single-stranded cDNA was synthesized. Amplification of cDNA was performed in 20 μL reactions containing 10 μL of 2× Taq premix I (SolGent Co., Korea) and 2 μL of each primer (5 pmol). To ensure that equal amounts of cDNA were added to the PCRs, an internal standard was amplified using the β-actin gene oligonucleotides 5′-GTGGGCCGCTCTAGGCACCAA-3′ (forward) and 5′-CTCTTTGATGTCACGCACGATTTC-3′ (reverse). PCR amplification of the Bcl-xL gene was carried out using the following sequence-specific primers 5′-CACATAACCCCAGGGACCGCGTA-3′ (forward) and