Available online at www.sciencedirect.com
SCIENCE DIRECT®
Hearing Research 204 (2005) 127–139
HEARING RESEARCH
www.elsevier.com/locate/heares

Protective effect of T-type calcium channel blocker flunarizine on cisplatin-induced death of auditory cells

Hong-Seob So [a], Channy Park [a], Hyung-Jin Kim [a], Jung-Han Lee [a], Sung-Yeol Park [a], Jai-Hyung Lee [a], Zee-Won Lee [b], Hyung-Min Kim [c], Federico Kalinec [d], David J. Lim [d], Raekil Park [a,*]

[a] *Vestibulocochlear Research Center and Department of Microbiology, Korea Basic Science Institute, Taejon 305-333, Korea*
[b] *Biomolecule Research Team, Korea Basic Science Institute, Taejon 305-333, Korea*
[c] *Department of Pharmacology, College of Oriental Medicine, Kyung Hee University, Seoul, Korea*
[d] *Gonda Department of Cell and Molecular Biology, House Ear Institute, 2100 West 3rd Street, Los Angeles, CA 90057, USA*

Received 29 July 2004; accepted 22 January 2005
Available online 11 March 2005

Abstract

Changes in intracellular Ca^{2+} level are involved in a number of intracellular events, including triggering of apoptosis. The role of intracellular calcium mobilization in cisplatin-induced hair cell death, however, is still unknown. In this study, the effect of calcium channel blocker flunarizine (Sibelium™), which is used to prescribe for vertigo and tinnitus, on cisplatin-induced hair cell death was investigated in a cochlear organ of Corti-derived cell line, HEI-OC1, and the neonatal (P2) rat organ of Corti explant. Cisplatin induced apoptotic cell death showing nuclear fragmentation, DNA ladder, and TUNEL positive in both HEI-OC1 and primary organ of Corti explant. Flunarizine significantly inhibited the cisplatin-induced apoptosis. Unexpectedly, flunarizine increased the intracellular calcium ($[Ca^{2+}]_i$) levels of HEI-OC1. However, the protective effect of flunarizine against cisplatin was not mediated by modulation of intracellular calcium level. Treatment of cisplatin resulted in ROS generation and lipid peroxidation in HEI-OC1. Flunarizine did not attenuate ROS production but inhibited lipid peroxidation and mitochondrial permeability transition in cisplatin-treated cells. This result suggests that the protective mechanism of flunarizine on cisplatin-induced cytotoxicity is associated with direct inhibition of lipid peroxidation and mitochondrial permeability transition.

Keywords: Cisplatin; Ototoxicity; Flunarizine; Organ of Corti

1. Introduction

Cisplatin, *cis*-diamminedichloroplatinum II; CDDP, is an extensively used chemotherapeutic agent in treatment of a broad spectrum of tumors (Fram, 1992). However, cisplatin often causes irreversible and progressive sensorineural hearing loss as well as tinnitus as serious side effects. Many studies have revealed that cisplatin primarily damages the outer hair cells (OHCs), especially located in the basal and middle turns of the cochlea with sporadic loss of inner hair cells (IHCs) (Estrem et al., 1981; Laurell and Bagger-Sjoback, 1991). Also, it could induce the

Abbreviations: NAC, *N*-acetylcysteine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; DMEM, Dulbecco's modified essential medium; FBS, fetal bovine serum; OHC, outer hair cells; IHC, inner hair cells; GSH, glutathione; TUNEL, Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling

* Corresponding author. Present address: Department of Microbiology, Wonkwang University School of Medicine, 344-7 Shinyong-dong Iksan, Jeonbuk 570-749, Korea (South). Tel.: +82 63 850 6777; fax: +82 63 852 0220.
E-mail address: rkpark@wonkwang.ac.kr (R. Park).

0378-5955/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.heares.2005.01.011

degeneration of the stria vascularis (Tange and Vuzevski, 1984; Tsukasaki et al., 2000) and a significant decrease in the number of spiral ganglion cells (Gabaizadeh et al., 1997). Although the exact mechanism involved in cisplatin ototoxicity still remains obscure, many evidences, including ROS generation to interfere the antioxidant defense systems of hearing organs, have been accumulating, in part (Appenroth and Winnefeld, 1993; Clerici et al., 1996; Gabaizadeh et al., 1997; Hannemann and Baumann, 1988; Kopke et al., 1997). Many attempts of antioxidant therapies (Campbell et al., 2003; Li et al., 2001; Rybak and Kelly, 2003; Teranishi et al., 2001; Teranishi and Nakashima, 2003) have been tried to ameliorate the ototoxicity of cisplatin in experimental animals in vivo.

Changes in intracellular Ca^{2+} ($[Ca^{2+}]_i$) concentration stimulate a number of intracellular events including triggering of apoptosis (Rizzuto et al., 2003). In normal condition, increases in $[Ca^{2+}]_i$ also play a pivotal role in regulating hair cell functions (Blanchet et al., 1996; Dulon et al., 1998; Yamoah et al., 1998). It is involved in both mechanotransduction at cell's apex and release of neurotransmitter at its base. Also, several studies have showed that treatment of gentamicin and cisplatin resulted in changes of $[Ca^{2+}]_i$. In particular, gentamicin blocked the depolarization-induced increase of $[Ca^{2+}]_i$ in isolated hair cells (Dulon et al., 1991) and voltage- and depolarization-mediated calcium current in dissociated outer hair cells (Saito et al., 1991; Yamamoto et al., 1994). However, the roles of $[Ca^{2+}]_i$ in cisplatin-induced hair cell death are less known. In this study, we examined the effect of various calcium channel modulators on cisplatin-induced death of auditory cells. Herein, our data demonstrate that flunarizine, a T-type calcium channel blocker, protects auditory cells from cisplatin cytotoxicity.

2. Experimental procedures

2.1. Reagents

Cisplatin, flunarizine, pimozide, nefedipine, nicardipine, dantrolene, *N*-acetylcysteine (NAC), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (Sigma, St. Louis, MO). Genomic DNA purification kit was obtained from Promega (Promega, Medison, WI). The plastic culture wares were bought from Falcon Inc (Becton Dickinson Biotech, Lincoln, IL). Dulbecco's modified essential medium (DMEM), fetal bovine serum (FBS), and other tissue culture reagents were obtained from Life Technologies Inc (Grand Island, NY).

2.2. Cell culture and viability

The establishment and characterization of the conditionally immortalized HEI-OC1 was described by Kalinec et al. (2003). HEI-OC1 was maintained in high glucose Dulbecco's modified Eagle medium (DMEM) containing 10% FBS and 50 U/ml interferon-γ (Genzyme, Cambridge, MA). For the experiments described below, HEI-OC1 was cultured under permissive conditions: 33 °C, 5% CO_2 in DMEM supplemented with 10% FBS. Cells (3×10^4 cells/each well of 24-well plate) were incubated with varying concentrations of cisplatin for 48 h. To determine the cell viability, MTT (0.5 mg) was added to 1 mL of cell suspension for 4 h. After three washes of cells with phosphate buffered saline (PBS, pH 7.4), the insoluble formazan product was dissolved in DMSO. Optical density (OD) of each culture well was measured using Microplate reader (Titertek Multiskan, Flow Laboratories) at 590 nm. The OD in control cells was taken as 100% of viability.

2.3. Hoechst 33258 staining of nuclei

The nuclei of HEI-OC1 were stained with chromatin dye, Hoechst 33258. Cells were fixed with 3.7% paraformaldehyde for 10 min at room temperature (RT), washed twice with PBS, and then incubated with 10 μM Hoechst 33258 in PBS at RT for 30 min. After three washes, cells were observed under fluorescence microscope (IX71, Olympus, Japan).

2.4. Mitochondrial membrane permeability transition (MPT)

Mitochondria of HEI-OC1 were stained with Rhodamine-123. Cells were incubated with 5 μg/ml Rhodamine-123 at 37 °C for 30 min. After incubation, cells were washed three times with PBS and observed under fluorescence microscope. Alterations in MPT showed a diffused form of a green fluorescence in the cytosol as well as in the nuclei.

2.5. Preparation of genomic DNA and electrophoresis

Genomic DNA was isolated by Wizard genomic DNA purification kit (Promega) and purified by serial ethanol precipitations. Five microgram of isolated genomic DNA was subjected onto 1.5% of agarose gel electrophoresis at 50 V for 3 h. DNA was visualized by staining with ethidium bromide under UV light.

2.6. Caspase activity assay

Whole cell lysate was prepared in a lysis buffer (1% Triton X-100, 0.32 M sucrose, 5 mM EDTA, 1 mM PMSF, 1 μg/ml leupeptin, 1 mM DTT, 10 mM Tris–HCl, pH 8.0) on ice for 30 min and centrifuged at 20,000*g* for 15 min. Equal amount of total protein was quantified by BCA protein quantification kit (Sigma) in each lysate. Catalytic activity of caspase-3 from cell lysate was measured by