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Calcium imaging in gentamicin ototoxicity: Increased intracellular calcium relates to oxidative stress and late apoptosis

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ABSTRACT

Objectives: To estimate intracellular calcium changes in gentamicin (GM) ototoxicity using calcium imaging. To investigate GM-induced physiologic changes in auditory cells including cell viability, apoptosis, and oxidative stress.
Methods: Varying concentrations of GM were applied to the HEI-OC1 cochlear cell line. Calcium imaging tracked changes in intracellular calcium concentration during GM cytotoxicity. Cell viability and intracellular reactive oxygen species (ROS) levels also were measured.
Results: Little change in calcium levels occurred in HEI-OC1 cells exposed to less than 35 mM GM. However, calcium rose continuously in cells exposed to more than 60 mM GM. With administration of intermediate concentrations of 40 or 50 mM GM, calcium increased variably in different cells, returning to baseline in some cases, or rising continuously in others. Upon increase of GM concentration, intracellular calcium concentration and ROS were increased, and cell viability was decreased due to late apoptosis.
Conclusion: This study shows that GM increased intracellular calcium, ROS, and late apoptosis of HEI-OC1 cells derived from cochlear tissue. Increase of intracellular calcium is related to GM-induced apoptosis and oxidative stress. Calcium imaging can be used to determine change of intracellular calcium concentrations and apoptosis in GM ototoxicity.

1. Introduction

Currently, intratympanic gentamicin (GM) is a major treatment modality for intractable Meniere's disease and other conditions involving peripherally initiated vertigo [1–3]. A major complication of intratympanic GM revealed by these studies is the risk of sensori-neural hearing loss (SNHL), which occurs in 20% of patients [2,4,5]. Through trial and error, vertigo control has improved, but the risk of SNHL is still the primary problem with intratympanic GM therapy. A major complicating factor in the occurrence of SNHL is the concentration of intratympanic GM. The concentration of injected GM varies from 18 mg/mL to 80 mg/mL, and a preparation at a concentration of 26.7 mg/mL (55.9 mM) is used commonly [2,4,5]. However, little is known regarding the maximal safe dose of GM, in particular its actions on auditory hair cells whose damage underlies SNHL.

GM ototoxicity can be induced by both apoptosis and necrosis pathway, and GM induced apoptosis is related to intracellular calcium increase. In endoplasmic reticulum (ER), GM blocks ribosomal protein synthesis, and causes ER stress, the unfolded protein response (UPR), and cell cycle arrest. Finally the cell undergoes apoptosis, which is mediated by the classical route of calpains and caspase [6] activated by the release of Ca^{2+} from the ER [7]. Meanwhile, GM enters into cells via endocytosis mediated by the megalin/cubilin complex. The drug mostly accumulates in the lysosomes and ER. In the lysosomes, GM produces membrane destabilization and lysosomal aggregation. Eventually GM causes proteolysis and cellular necrosis through cathepsins [7].

The production of mitochondrial reactive oxygen species (ROS) is caused by GM actions on the respiratory chain [7]. GM also reduces glutathione and superoxide dismutase, important antioxidants in cells. The accumulated GM is released into the cytosol where it acts on mitochondria and activates the mitochondrial pathway of apoptosis, produces ROS and reduces the ATP reserve [7]. Oxidative stress induced by GM causes an increase in calcium influx through the L-type calcium channel [8]. Increase of calcium causes further increases in superoxide production by the mitochondria, which causes further oxidation of L-type calcium channels and further calcium influx into the cells [8,9]. Thus, GM causes the production of ROS and calcium overload leading to cellular apoptosis.

Calcium overload can disturb various calcium sensitive targets with key cellular functions [10]. Calcium deregulation also disturbs

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the homeostasis of auditory cells [11]. It has long been recognized that a disruption of calcium homeostasis is related to cellular damage [12]. In GM ototoxicity, calcium overload is induced by both apoptotic process and oxidative stress, and finally plays a role in cellular death [6,13].

The purpose of this study is to measure changes in intracellular calcium concentration in GM ototoxicity using calcium imaging. We also investigate cell viability, ROS, and the pattern of apoptosis. Increases in intracellular calcium concentration can indirectly reflect the severity of GM ototoxicity and oxidative stress. Tracking these physiological changes in GM ototoxicity can help to determine the severity of GM ototoxicity, and can be used as a reference about choice of a safe and suitable intratympanic GM dose range for intractable vertigo control.

2. Materials and methods

2.1. HEI-OC1 cell culture

The HEI-OC1 cell line was kindly provided by F. Kalinec (House Ear Institute, Los Angeles, CA, USA). HEI-OC1 cells express several molecular markers which are characteristic of organ of Corti sensory cells, and HEI-OC1 cells are extremely sensitive to ototoxic drugs [14]. Therefore, the HEI-OC1 cell line can be a useful model for the study of ototoxic drugs such as aminoglycoside and cisplatin [15,16]. The cells used in this study were maintained in DMEM with 10% FBS at 33 °C under 10% CO_2 in air. Commercial GM solution (Choongwae Pharma Co., 40 mg/mL, molecular weight: 477.6, 83.7 mM) was used in this study, and was diluted to concentrations ranging from 30 mM to 83.7 mM.

2.2. Measurement of intracellular calcium levels

The intracellular calcium concentrations in HEI-OC1 cells were measured with Fura-2 AM (8 μM) in L-15 media, with digital microscopy (Universal; Carl Zeiss, Inc., Thornwood, NY, USA). Fura-2 is a 'ratiometric' dye that can report calcium concentration independent of dye concentration or image thickness. A field of cells was monitored by sequential dual excitation at 352 and 380 nm, and the image ratio was analyzed by a method described previously [12]. Images were acquired every 3 s, and calcium concentrations for negative control (baseline) were measured in the first 10 cycles (30 s).

The cells on the coverslip were exposed to various concentrations of GM between cycle 30 (90 s) and cycle 150 (450 s). The response to GM was measured from the period beginning with application of GM through application of ionomycin (positive control) at cycle 150. Changes in intracellular calcium concentrations were calculated using 10 cycles within the final 1/3 of measurements in the presence of GM. At each dose of GM tested, the baseline response was compared to this mean calcium indicator response. Each result was analyzed using a paired sample *t* test.

After GM exposure, the cells were finally exposed to 1 μM of the ionophore ionomycin from cycle 150 to cycle 180 (30 cycles; 90 s). The calcium concentration in ionomycin was taken as the maximal response. Each set of images for calcium measurement also included a bright field image of the field of cells under study. About twenty of the cells that had an average diameter (long and short axis) of over 15 μm were analyzed. A schematic summary of calcium imaging is presented as Fig. 1.

2.3. Cell viability study (MTT assay)

The uptake and conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazdiumbromide (MTT, Sigma, St. Louis, MO, USA) to crystals of dark violet formazan depend on cell viability. Thus, HEI-OC1 cells (10^5 cells/well of 24-well plate) were incubated with various concentrations of GM for 24 h, and the dose- and time-dependent effects of GM were measured using the MTT assay. For the MTT assay, 50 μL of MTT solution (0.25 mg) was added to 0.5 mL of cell suspension, and the plates were then incubated for 4 h at 33 °C in 10% CO_2. The insoluble formazan crystals were centrifuged, and the pellets were dissolved by the addition of DMSO (500 μL/well). The optical density was measured using a microplate reader at 570 nm (Spectra Max, Molecular Devices, USA).

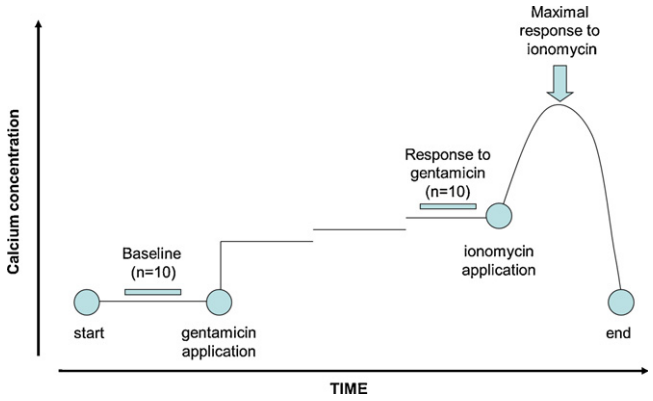

Fig. 1. Schematic summary of calcium imaging. The intracellular calcium concentrations in HEI-OC1 cells were measured with Fura-2. Baseline was determined by using 10 imaging cycles within the first 30 cycles (3 s per cycle). After baseline measurement, the cells were exposed to various concentrations of gentamicin (GM). Response to GM and changes in intracellular calcium concentrations were calculated using 10 cycles within the final 1/3 of calcium concentration measurements for GM. Finally, the cells were exposed to ionomycin, and the maximal response for ionomycin was determined by using the maximum value of intracellular calcium concentration after ionomycin application.

2.4. Measurement of intracellular reactive oxygen species production and Hoechst 33258 staining

The nuclei of HEI-OC1 cells were stained with the chromatin dye Hoechst 33258 (Sigma, St. Louis, MO, USA). Cells were washed twice with phosphate buffered saline (PBS) and fixed with 3.7% glutaraldehyde for 10 min at room temperature (RT). After fixation, they were washed twice with PBS and incubated with 10 μg/mL Hoechst 33258 for 10 min at RT in the dark. After two washes, the cells were observed under an inverted fluorescence microscope (BX61, Olympus, Japan). The intracellular ROS level was measured using a fluorescent dye, 2′,7′-dichlorofluorescein diacetate (DCFH-DA; Eastman Kodak, Rochester, NY). In the presence of an oxidant, DCFH is converted into highly fluorescent 2′,7′-dichlorofluorescein (DCF). For the assay, HEI-OC1 cells were cultured overnight on coverslips, and then treated with GM for 24 h. Cells were washed twice with serum-free medium without phenol red then incubated with 5 μM DCFH-DA in serum-free medium without phenol red for 10 min at 33 °C. After three washes with serum-free medium without phenol red, cells were fixed with 3.7% glutaraldehyde for 10 min at RT. Cells were incubated with 10 μg/mL Hoechst 33258 for 5 min at RT in the dark. After washing twice with PBS, the samples were immediately observed using an excitation wavelength of 387 nm, and then the fluorescent intensity was measured using an excitation wavelength of 485 nm and an emission wavelength of 538 nm with a long term real-time live cell imaging system (LAMBDA DG-4, Sutter Instr., Novato, USA). Imaging of 100 μM H_2O_2 was used as a positive control, and saline as a negative control.