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Vancomycin induces reactive oxygen species-dependent apoptosis via mitochondrial cardiolipin peroxidation in renal tubular epithelial cells

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ABSTRACT

Vancomycin (VCM) is a first-line antibiotic for serious infections caused by methicillin-resistant *Staphylococcus aureus*. However, nephrotoxicity is one of the most complaint in VCM therapy. We previously reported that VCM induced apoptosis in a porcine proximal tubular epithelial cell line (LLC-PK1), in which mitochondrial complex I may generate superoxide, leading to cell death. In the present study, VCM caused production of mitochondrial reactive oxygen species and peroxidation of the mitochondrial phospholipid cardiolipin that was reversed by administration of the mitochondrial uncoupler carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP). FCCP also significantly suppressed VCM-induced depolarization of the mitochondrial membrane and apoptosis. Moreover, the lipophilic antioxidant vitamin E and a mitochondria-targeted antioxidant, mitoTEMPO, also significantly suppressed VCM-induced depolarization of mitochondrial membrane and apoptosis, whereas vitamin C, n-acetyl cysteine, or glutathione did not provide significant protection. These findings suggest that peroxidation of the mitochondrial membrane cardiolipin mediated the VCM-induced production of intracellular reactive oxygen species and initiation of apoptosis in LLC-PK1 cells. Furthermore, regulation of mitochondrial function using a mitochondria-targeted antioxidant, such as mitoTEMPO, may constitute a potential strategy for mitigation of VCM-induced proximal tubular epithelial cell injury.

1. Introduction

Vancomycin (VCM) is a glycopeptide antibiotic used as a first-line therapy for serious infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA). However, nephrotoxicity is one of the most common complaint associated with VCM therapy. Recent guidelines have recommended maintaining higher VCM trough levels (15–20 mg/l) and achieving an area under the curve/minimum inhibitory concentration of ≥400 in the treatment of complicated MRSA infections (Rybak et al., 2009; Álvarez et al., 2016). However, higher VCM doses and trough concentrations could increase the likelihood of nephrotoxicity, particularly in patients at high risk for acute kidney injury (Álvarez et al., 2016; Elyasi et al., 2012; Wong-Beringer et al., 2011). A recent meta-analysis of 15 clinical studies showed that VCM-associated nephrotoxicity is significantly more prevalent in patients with high VCM trough levels (≥15 mg/l) than in patients with low trough levels (< 15 mg/l) (van Hal et al., 2013). Therapeutic drug monitoring is useful for prevention of nephrotoxicity induced by VCM; however, it is difficult to ensure the clinical efficacy of VCM and simultaneously decrease nephrotoxicity in clinical settings. Although the precise mechanisms underlying VCM-associated nephrotoxicity are largely unknown, renal tubular cell damage and oxidative stress have been implicated in the pathogenesis (Cetin et al., 2007; Oktem et al., 2005; Dieterich et al., 2009).

We previously reported that VCM induced apoptotic injury in the porcine proximal tubular epithelial cell line LLC-PK1 (Arimura et al., 2012). In particular, VCM increased intracellular reactive oxygen species production, leading to depolarization of the mitochondrial membrane and activation of caspase-9 and −3/7. Moreover, we demonstrated that mitochondrial respiratory chain complex I may play

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a key role in VCM-induced superoxide generation and proximal tubular epithelial cell apoptosis (Arimura et al., 2012). The present study was designed to clarify the mechanisms underlying VCM-induced proximal tubular cell damage and to determine whether regulation of mitochondrial function can mitigate VCM-induced renal tubular epithelial cell injury.

2. Materials and methods

2.1. Chemicals

VCM hydrochloride, α-tocopherol (vitamin E), n-acetyl cysteine (NAC), glutathione (GSH), and sodium azide were purchased from Wako Pure Chemicals (Osaka, Japan). Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazine (FCCP), rotenone, 3-nitropropionic acid, and anti-β-actin monoclonal antibody were obtained from Sigma-Aldrich (St. Louis, MO, USA). Antimycin A was obtained from LKT Laboratories, Inc. (St. Paul, MN, USA). Ascorbic acid (vitamin C) was purchased from Nacalai Tesque (Kyoto, Japan). Horseradish peroxidase-conjugated goat anti-mouse antibodies were obtained from Jackson Immuno Research Laboratories, Inc. (West Grove, PA, USA). Anti-cytochrome c monoclonal antibody was purchased from Bio Vision, Inc. (Milpitas, CA, USA). 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA), MitoSOX Red, and 10-N-nonyl acridine orange (NAO) were purchased from Life Technologies (Carlsbad, CA, USA). Hoechst 33342 was obtained from Dojindo Laboratories (Kumamoto, Japan). (2-(2,2,6,6-Tetramethylpiperidin-1-oxyl-4-ylamino)−2-oxoethyl)triphenylphosphonium chloride (MitoTEMPO) was purchased from Enzo Life Sciences, Inc. (Farmingdale, NY). Caspase inhibitors, namely, zDEVD-fmk (selective for caspase-3) and zLEHD-fmk (selective for caspase-9), were purchased from Merck Millipore (Billerica, MA, USA).

2.2. Cell culture

The porcine proximal tubular epithelial LLC-PK1 cell line was obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were maintained in Medium 199 (MP Biomedicals, lrvine, CA, USA) containing 10% fetal bovine serum under an atmosphere of 5% CO_2 at 37 °C. Cells were seeded at a density of 2.0×10^4 cells/cm^2 onto cell culture dishes or plates and used for experiments on the following day, when they had reached 70–80% confluence.

2.3. Cell viability

Cell viability was assessed using Presto Blue cell viability reagent (Life Technologies) according to the manufacturer's instructions. Presto Blue is reduced from resazurin, a blue compound with no intrinsic fluorescence, to resorufin, which is red in color and highly fluorescent upon entering living cells (Lall et al., 2013). Cells were seeded onto 96-well plastic plates (Corning, Inc., Corning, NY, USA). After VCM treatment, cells were washed with phosphate-buffered saline (PBS) and incubated with 90 μl of serum-free medium and 10 μl of Presto Blue reagent for 1 h at 37 °C in humidified air supplemented with 5% CO_2. The incubation medium was transferred to 96-well flat-bottom black plates (Nalge Nunc International, Penfield, NY, USA). Resorufin fluorescence was measured at an excitation wavelength of 544 nm and an emission wavelength of 590 nm using a microplate reader (Flex station 3; Molecular Devices, Sunnyvale, CA, USA).

2.4. Western blot analysis of cytochrome c

Release of cytochrome c from the mitochondria to the cytoplasm was assessed by western blot. Cells were lysed and the cytosolic fractions were isolated using a mitochondria/cytosol-fractionation kit (Bio Vision) according to the manufacturer's instructions. Briefly, cells (~2.0×10^6) were collected by cell scraper and centrifuged at 150*g* for 5 min. Cell pellets were homogenized and centrifuged at 700*g* for 10 min. The supernatant was centrifuged at 10,000*g* for 30 min to isolate the mitochondrial fraction. Proteins from the cytosolic fractions were subjected to 13.5% SDS-PAGE and transferred electrophoretically to polyvinylidene difluoride membranes. The membranes were blocked in Tris-buffered saline Tween-20 containing 5% bovine serum albumin or non-fat dry milk for 1 h at room temperature with agitation. The membrane was incubated overnight at 4 °C with an anti-cytochrome c or β-actin antibody (1:5000) and then incubated for 1 h with anti-mouse IgG horseradish peroxidase (1:5000). To ensure equal protein loading, β-actin (cytosolic fraction) was used as an internal control. Immunoreactivity was detected using enhanced chemiluminescence (Perkin Elmer, Waltham, MA, USA) and a chemiluminescence detector (LAS 4000; GE Healthcare Life Sciences, Pittsburgh, PA, USA).

2.5. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay

The quantification of apoptotic cell death was assessed by TUNEL staining, as described previously (Arimura et al., 2012). Briefly, after exposure to VCM, cells were permeabilized with 70% ethanol overnight at −20 °C. The TUNEL assay was performed using the Dead End fluorometric system (Promega Corp., Madison, WI, USA) according to the manufacturer's instructions. The fluorescent cells were quantitatively analyzed using flow cytometry (Becton-Dickinson, Franklin Lakes, NJ, USA), and Cell Quest software (Becton-Dickinson).

2.6. Determination of intracellular reactive oxygen species and mitochondrial superoxide production

Intracellular reactive oxygen species and mitochondrial superoxide were determined using carboxy-H_2DCFDA fluorescence labeling or a selective mitochondrial superoxide probe, MitoSOX red (Life Technologies) as described previously. Briefly, cells were seeded onto 12-well plastic plates (Becton-Dickinson). At 24 h after seeding, the cells were exposed to VCM for the indicated duration. The cells were washed twice with PBS and treated with carboxy-H_2DCFDA (50 μM) for 1 h or MitoSOX Red (2 μM) for 15 min at 37 °C. Cells were trypsinized and resuspended in PBS, and then analyzed by flow cytometry (Becton-Dickinson). At least 10,000 cells were collected for each sample, and data were analyzed by Cell Quest software (Becton-Dickinson).

2.7. Assay for caspase-3/7 activity

Caspase-3/7 activities were assessed by the Apo-ONE homogeneous caspase-3/7 assay kit (Promega) according to the manufacturer's instructions. Briefly, after exposure to VCM, cells were washed with PBS and incubated with caspase substrate for 1 h at room temperature. Caspase-3/7 activities were measured at an excitation wavelength of 490 nm and an emission wavelength of 530 nm using a fluorescence plate reader (MTP-601F; Hitachi High-Technologies Corp., Tokyo, Japan).

2.8. Assessment of cardiolipin peroxidation

Cardiolipin peroxidation was assessed using NAO, which is a cell-permeating fluorescent indicator that accumulates in mitochondria and binds to cardiolipin. Peroxidation of cardiolipin causes a decrease in the binding affinity of NAO to cardiolipin, resulting in decreased fluorescence (Ferlini and Scambia, 2007). In brief, after exposure to VCM, cells were washed twice with PBS and loaded with NAO (30 nM) for 15 min at 37 °C. Cells were visualized using an all-in-one florescence microscope (BZ-9000; Keyence, Tokyo, Japan). The mean