Tetramethylpyrazine protects rat renal tubular cell apoptosis induced by gentamicin

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Abstract

Background. Gentamicin, a widely used antibiotic for the treatment of bacterial infection, can cause nephrotoxicity. Tetramethylpyrazine (TMP) is a compound purified from the rhizome of Ligusticum wallichi (Chuanxiong) and has been found to protect against ischaemia–reperfusion injury, nephritis and alcohol-induced toxicity in rat kidneys.

Methods. We used rat renal tubular cells (RTCs), NRK-52E, in this study. The cytotoxicity of gentamicin was checked with transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) staining, and the generation of reactive oxygen species was measured using the fluorescent probe 2,7-dichlorofluorescein. We evaluated several apoptotic parameters: cleaved caspase levels, tumour necrosis factor (TNF-α) excretion and nuclear factor Kappa B (NF-κB) activity. We also examined the TMP protective effect on gentamicin-induced apoptosis in rat kidneys.

Results. The results of this study showed that gentamicin was found to markedly induce apoptosis in NRK-52E cells in a dose-dependent manner; that TMP expressed a dose-dependent protective effect against gentamicin-induced apoptosis; that pre-treatment of the cells with 50 or 100 μM of TMP effectively decreased the reactive oxygen species formation induced by gentamicin; that TMP was found to inactivate the gentamicin-stimulated activities of caspase-3, caspase-8 and caspase-9, to inhibit gentamicin-induced release of cytochrome c, as well as to raise the expression of Bcl-xL; that TMP inhibited the gentamicin-induced TNF-α excretion, and inactivated the transcription factor NF-κB; and that the TMP treatment significantly reduced apoptotic injury in rat RTCs.

Conclusions. Based on the results of this study, we suggest that TMP can attenuate gentamicin-induced oxidative stress and apoptotic injury in rat RTCs, and that its character may have therapeutic potential for patients with renal diseases.

Keywords: apoptosis; gentamicin; renal tubular cell (RTC); tetramethylpyrazine (TMP)

Introduction

Gentamicin is a cheap, important and widely-used antibiotic of aminoglycoside for the treatment of Gram-negative bacterial infection. But nephrotoxicity is the main side effect that seriously limits its use. Gentamicin treatment causes acute renal failure with acute tubular necrosis in about 20% of the patients [1]. Gentamicin has been found to bind with acidic, negatively charged phospholipid-binding sites at the level of the brush border membrane through a process in which megalin plays a role [2]. The intravenously administered gentamicin is almost entirely eliminated by the kidney, but a small, toxic portion is selectively reabsorbed and accumulates in the proximal renal tubular cells (RTCs) [1,2]. Lysosomes are the first and obvious site of sequestration and accumulation of gentamicin in proximal tubules. Gentamicin in lysosomes can be released into cytoplasm through drug overloading, direct permeabilization of the lysosomal membrane, or retrograde trafficking through the Golgi apparatus and the endoplasmic reticulum, resulting in cell death [3–5].

Inducing apoptosis is an important cytotoxic mechanism in gentamicin-treated proximal RTC and mesangial cells [4,6,7]. Servais et al. [3,4] reported concentration-dependence of the onset of gentamicin-induced apoptosis in the 1–3 mM range.
Reactive oxygen species (ROS) are important mediators of gentamicin-induced apoptosis [8]. ROS generation is often responsible for the mitochondria-mediated signalling pathway of apoptosis. Gentamicin can induce apoptosis in LLC-PK1 (Lilly Laboratories, Culture-Pig Kidney type 1) cells through triggering the mitochondrial pathway and activating caspase-3 [3]. In mammalian cells, a major caspase activation pathway is the cytochrome c-initiated pathway. In this pathway, various apoptotic stimuli cause cytochrome c release from mitochondria, to induce a series of biochemical reactions, resulting in activating caspase to cause subsequent cell death [9]. Cytochrome c release is known to be regulated by Bcl-2 family proteins, including Bcl-2 and Bcl-xL, they bind to the mitochondrial outer membrane and block cytochrome c efflux [10]. In addition, the death receptor-mediated pathway is also an important apoptosis signalling pathway, which requires binding of a ligand to a death receptor on the cell surface: tumour necrosis factor (TNF) and Fas ligand receptors [11]. However, the underlying molecular mechanisms of gentamicin-induced apoptosis still remain poorly defined.

Tetramethyldiprazine (TMP, also named ligustazine) is purified from the rhizome of *Ligustrum wallichii* (Chuanxiong). It is a widely used active ingredient in the Chinese herbal medicine to treat coronary artery and ischaemic cerebral vascular diseases [12,13]. Besides vasodilatory actions and antiplatelet activity, TMP has strong effects to remove oxygen free radicals, and the ROS decrement may be associated with increased activities of superoxide dismutase (SOD), catalase and glutathione peroxidase [14–16]. In the studies of rat kidney, TMP was found to prevent ischaemia–reperfusion injury, nephritis and alcohol-induced toxicity [16–18]. TMP also has hepatoprotective and therapeutic effects on acute econazole-induced liver injury [19]. Such findings may indicate that TMP can protect and treat renal diseases. In the present study, we intended to investigate the TMP influence on apoptotic pathways, and to further evaluate the protective effect of TMP on gentamicin-induced apoptotic injury in rat RTCs.

**Materials and methods**

**Reagents**

Dulbecco’s modified Eagle’s medium (DMEM), fetal calf serum and tissue culture reagents were obtained from Life Technologies, Inc., (Gaithersburg, MD, USA). We purchased TMP from Aldrich (St Louis, MO, USA), and all other chemicals of reagent grade were obtained from Sigma (St Louis, MO, USA).

**Cell culture**

We purchased rat proximal RTCs (NRK-52E) from the Bioresource Collection and Research Center (Taiwan), and cultured them in DMEM culture medium supplemented with antibiotic/antifungal solution and 10% fetal bovine serum. They were grown until the monolayer became confluent. The medium for the cultured cells was then changed to the serum-free medium, and the cells were incubated overnight before the experiment.

**TUNEL stain**

Gentamicin-mediated apoptosis in NRK-52E cells was detected by enzymatic labelling of DNA strand breaks using terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labelling (TUNEL) stain. As described previously [20], the TUNEL stain was performed with a cell death detection kit (Roche, Mannheim, Germany). To reveal total nuclei, we stained the same slides with DAPI (4′,6-diamidino-2-phenyindole) (1 μg/ml) in phosphate-buffered saline (PBS) plus 0.5% 1,4-diazobicyclo[2.2.2]octane.

**Western blot analysis**

We applied 30 μg of NRK-52E lysate proteins to each lane and analysed them with western blots. Cytosol cytochrome c was extracted by using cytochrome c Release Apoptosis Assay Kit (Calbiochem Inc., Darmstadt, Germany). We purchased the antibodies of caspase-3, caspase-8, caspase-9, cytochrome c, Bcl-xL and nuclear factor-kappa B (NF-κB) from BD Laboratories (San Jose, CA, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA), and diluted to 1:1000 for the assay. We used peroxidase-conjugated anti-rabbit or anti-mouse IgG (1:5000 dilution) as the second antibody to detect caspase-3, caspase-9, cytochrome c, Bcl-xL and NF-κB bands by enhanced chemiluminescence (Amersham Biosciences, Pittsburgh, PA, USA).

**Determination of the cellular uptake of gentamicin**

We cultured the NRK-52E cells in 6-cm plates with 3 mM gentamicin for different time periods with or without 100 μM TMP pre-treatment, washed them three times with PBS buffer, and then lysed them with radio-immunoprecipitation assay buffer. The 0-h groups were washed immediately after gentamicin treatment. The concentration of gentamicin in each sample was detected using gentamicin EIA kits according to instructions provided by the manufacturers (Euro-Diagnostica B.V., Arnhem, The Netherlands).

**The detection of intracellular ROS**

Before the chemical treatment, we incubated NRK-52E cells in a culture medium containing a fluorescent dye, 2′,7′-dichlorofluorescein (DCF) of 30 μM for 30 min to establish a stable intracellular level of the probe. We kept the same concentration of DCF during the chemical treatment. Subsequently, the cells were washed with PBS, removed from the Petri dishes by scraping, and measured for DCF fluorescence intensity. We determined the DCF fluorescence intensity of the cells by fluorescence spectrophotometer with excitation and emission wavelengths at 475 and 525 nm, respectively. The cell number in each sample was counted and utilized to normalize the fluorescence intensity of DCF. We also performed a chemiluminescence assay of superoxide production. NRK-52E cells were lysed after drug treatment with a lysis buffer containing lucigenin (200 μM).
Readings were begun immediately upon addition of the lysis buffer. We used samples with the addition of SOD (1.0 × 10^5 U/l) as blank controls. Each reading was recorded as single photon counts by using a microplate scintillation counter (Topcount, Packard Instrument Co., Meriden, CT, USA).

**Determination of TNF-α**

We cultured NRK-52E cells on 10-cm plates with 2ml medium in each plate, pre-treated them with or without TMP for 30 min and then with gentamicin at 3 mM for 8 h. The cultured medium was collected and analysed using the mouse TNF-α ELISA kit according to instructions provided by the manufacturers (RayBiotech, Inc., Norcross, GA, USA).

**Electrophoretic mobility shift assay (EMSA)**

To prepare nuclear protein extracts, we washed cultured NRK-52E with cold PBS and then immediately removed them by scraping in PBS. After centrifugation of the cell suspension at 2000 rpm, the cell pellets were resuspended in cold buffer A (containing KCl 10 mM, EDTA 0.1 mM, DTT 1 mM and PMSF 1 mM) for 15 min. We lysed the cells by adding 10% NP-40 and then centrifuged them at 6000 rpm to obtain pellets of nuclei. The nuclear pellets were resuspended in cold buffer B (containing HEPES 20 mM, EDTA 1 mM, DTT 1 mM and PMSF 1 mM and NaCl 0.4 mM) vigorously agitated and then centrifuged. The supernatant containing the nuclear proteins was used for the western blot assay or stored at −70 °C until used. We purchased a double-stranded oligonucleotide containing a high affinity sequence for NF-κB from the mouse kappa-light chain enhancer (5'AGC TTC AGA GAC TTT CCG AGA GG3'). The oligonucleotide was end-labelled with[^32]P ATP. Extracted nuclear proteins (10 μg) were incubated with 0.1 ng[^32]P-labelled DNA for 15 min at room temperature in 25 μl binding buffer containing 1 μg poly (dI-dC). We electrophoresed the mixtures on 5% non-denaturing polyacrylamide gels. Gels were dried and imaged by autoradiography.

**Animals and treatments**

We purchased male Sprague-Dawley rats weighing 180–200 g from the Laboratory Animal Center, Taiwan University, (Taiwan). Before and during treatment, we housed animals in a central facility and submitted them to a 12-h light–dark cycle, and provided them with regular rat chow and tap water. The experimental group animals (n = 6) for gentamicin treatment received intraperitoneal (IP) injection with gentamicin (20 mg/kg/day) for 7 days. The group animals (n = 6) for gentamicin and TMP treatment received IP injection with TMP (80 mg/kg/day) 30 min before the gentamicin treatment, each time. Control group rats received a continuous infusion of 0.9% (w/v) saline throughout the gentamicin-treated period. Kidneys were harvested by laparotomy and sectioned for in situ TUNEL assays.

**In situ TUNEL assay**

We processed kidney slides with an ApopTag Fluorescein in situ apoptosis detection kit (CHEMICON International, Inc., CA, USA) according to the manufacturer’s instructions. Briefly, kidney tissue slides were pre-treated with proteinase K and H2O2, and incubated with the reaction mixture containing terminal deoxynucleotidyl transferase (TdT) and digoxigenin-conjugated dUTP for 1 h at 37°C, and mounted with mounting solution containing DAPI and observed with fluorescent microscope.

**Statistical analyses**

Data were presented as the mean ± SD, and groups were compared using the analysis of variance (ANOVA). The differences were considered significant if the P-values were <0.05.

**Results**

The gentamicin-induced apoptosis in rat RTC NRK-52E was detected using TUNEL staining. As shown in Figure 1A, the total nuclei on the slides were revealed by DAPI staining, and apoptotic nuclei were labelled by TUNEL. In dosage tests, exposure to ≥2 mM of gentamicin markedly increased apoptotic cells, and the cytotoxicity of gentamicin ascended along the increased concentration of gentamicin (Figure 1A and B). Subsequently, the protective effect of TMP against the apoptotic cytotoxicity was also examined. The pre-treatment of TMP significantly reduced gentamicin-induced apoptosis in a dose-dependent manner (Figure 1A and C). This result reveals that TMP protects NRK-52E cells from gentamicin-induced apoptosis.

We also monitored the influence of TMP on the apoptotic signalling pathway caused by gentamicin. The effect of TMP on gentamicin-induced activation of caspases was evaluated by western blotting using antibodies that recognize cleaved caspases (Figure 2). The increase of cleaved caspase-9 is associated with mitochondria-mediated signalling pathway in apoptosis. To evaluate the influence of TMP on the variations of mitochondria-mediated signalling molecules caused by gentamicin, such as cytochrome c and Bcl-xL, western blotting was carried out with the specific antibody against cytochrome c and Bcl-xL (Figure 3).

Furthermore, activating caspase-8 is often involved in death receptor-mediated apoptosis. TNF-α is one of the important ligands for death receptors. We monitored the gentamicin-induced excretion of TNF-α in NRK-52E cells to identify whether TNF-α was involved in gentamicin-induced apoptosis. As shown in Figure 4A, the excretion of TNF-α was lightly induced by gentamicin treatment in a dose-dependent manner. When TNF-α neutralization antibody was applied to neutralize TNF-α excreted from gentamicin-treated NRK-52E cells, the gentamicin-induced cleaved
caspase-8 was reduced (Figure 4B). With TMP pre-treatment, gentamicin-induced TNF-α excretion was reduced in NRK-52E cells (Figure 4C). These results reveal that TNF-α-mediated signalling-pathway plays a role in gentamicin-induced apoptosis, and is also inhibited by TMP treatment. We further monitored the activation of the transcription factor of NF-κB, in gentamicin-treated NRK-52E cells. EMSA was used to monitor the DNA-binding activity of nuclear NF-κB, which was responsible for the majority of NF-κB activity. As shown in Figure 5, the increase of DNA-binding activity of NF-κB by gentamicin was reduced significantly by TMP in a dose-dependent manner. It is possible that TMP is able to inhibit the gentamicin-induced activity of NF-κB to reduce TNF-α excretion.

To evaluate the mechanism of the protective effect of TMP on gentamicin-induced apoptosis, the influence of TMP on the cellular uptake of gentamicin was monitored. As shown in Figure 6A, there was a basal level of gentamicin in the 0-h groups. This may result from gentamicin adhering to the cell membranes of NRK-52E cells. After gentamicin treatment, the concentration of cytosol gentamicin reached a maximum within 30 min. However, TMP treatment
did not influence the concentration of cytosol gentamicin. These results reveal that TMP did not influence the cellular uptake of gentamicin. We next examined whether TMP prevents gentamicin-induced ROS formation because ROS are essential mediators in mitochondrial signalling pathways in apoptosis. As shown in Figure 6B, TMP significantly inhibited gentamicin-induced ROS formation under the treatment of 3 mM gentamicin for 24 h.

The protective effect of TMP on gentamicin-induced apoptosis was further proven in a rat animal model. Rats were treated with gentamicin (20 mg/kg/day) or saline as controls; the experimental groups were treated with TMP (80 mg/kg/day) in addition. At the end of the treatment period (7 days), kidneys were harvested by laparotomy and sectioned for in situ TUNEL assays. As shown in Figure 7, total nuclei in kidney sections were revealed as bright spots stained with DAPI. The scattered and bright nuclei stained by TUNEL staining could easily be detected over the entire cortex from gentamicin-treated animals, but rarely in the specimens of the controls and gentamicin-TMP-treated animals. Most of the TUNEL-labelled nuclei were seen in proximal RTCs. This result reveals that TMP inhibits the gentamicin-induced cell apoptosis in rat RTC in vivo.

**Discussion**

Since proximal RTC are the major targets of gentamicin-induced nephrotoxicity in both humans and animals [1], in this study, we focused on TMP influence on the gentamicin-induced apoptosis in those cells. The major findings of our study showed that TMP attenuated gentamicin-induced apoptotic injury in rat RTC NRK-52E (Figure 1). TMP did not influence the cellular uptake of gentamicin in NRK-52E cells, but reduced the gentamicin-induced ROS generation (Figure 6). ROS are important mediators and a
response for gentamicin-induced apoptosis [8]. Several studies have shown that TMP can remove oxygen free radicals, probably due to increased activities of SOD, catalase and glutathione peroxidase [14,15]. The inhibition on gentamicin-induced ROS generation may be an important TMP protective mechanism. Additionally, we monitored the apoptotic signals associated with gentamicin in NRK-52E cells, such as the activation of caspasas, the increased release of cytochrome c, the Bel-XL reduction and the induction of TNF-α excretion. The results of our study indicated that the apoptotic signals existed in gentamicin-treated NRK-52E cells, and were reversed by TMP treatment (Figures 2–4). Taken together, we suggest that TMP can protect gentamicin-treated rat RTC through the inhibiting apoptotic signalling pathways.

In the dose-dependent cytotoxicity assay, gentamicin concentration of ≥2 mM, significantly induced apoptosis in NRK-52E cells with 24-h treatment, as shown in Figure 1. To obtain obvious results, we used gentamicin concentration of 3 mM in the rest of the experiments. Some studies have revealed that the concentration range from 1 to 3 mM of gentamicin significantly induces apoptosis in LLC-PK1 and MDCK (Madin–Darby Canine Kidney) cells, and approximates the kidney levels of the drug that are reached in vivo during treatment [3,5,6,21]. As explained by El Mouedden et al. [6,21], this concentration range allows LLC-PK1 cells to obtain cellular and intralysosomal drug concentration as those observed in proximal tubular cells of rats receiving clinically relevant doses of gentamicin with apoptosis but not necrosis. In other words, the gentamicin concentration used in the in vitro study has a chance to exist in rat kidneys in vivo during treatment.

The TMP protective effect on gentamicin-induced apoptosis was also observed in our rat animal model (Figure 7). In our experiment, we gave IP gentamicin
20 mg/kg/day, which has been the equivalent maintenance dose for an adult patient with an estimated creatinine clearance of 90 ml/min [6]. In our study, the TMP protective dosage with 80 mg/kg/day was used IP 30 min before giving gentamicin. This dosage has been found to attenuate the necrosis and apoptosis in the tubules of ischaemia–reperfusion injury in murine kidney [18]. These findings may provide hints for future investigations in humans.

Caspase-dependent apoptotic signalling plays a major role in apoptotic injury induced by gentamicin. Caspase-3 is an executioner caspase that can be activated by caspase-9 in the mitochondrial pathway, or caspase-8 in the death receptor pathway [11]. Caspase-9 is activated from procaspase-9 by cytosolic cytochrome c [9]. The mitochondrial release of cytochrome c is regulated by Bcl-2 family proteins, including Bcl-2 and Bcl-xL which bind to the mitochondrial outer membrane and block cytochrome c efflux [10]. In our study, gentamicin was found to reduce the Bcl-xL expression which was reversed by TMP treatment (Figure 3). But the activation of caspase-8 implies that the death receptor pathway is involved in gentamicin-induced apoptosis in NRK-52E cells. TNF-α is also an important mediator in the death receptor pathway, and its gentamicin-induced excretion was inhibited by TMP treatment. Based on those findings of this study, we suggest that TMP can protect RTC from gentamicin-induced apoptotic injury through inhibiting both mitochondrial and death receptor pathways. Furthermore, NF-κB has a pro-apoptotic character due to its activating apoptotic genes, including TNF-α, Fas ligand, c-Myc and p53 [22,23]. The results of our study showed that TMP inhibited gentamicin-induced NF-κB activation in NRK-52E cells (Figure 5). Recent studies reveal that H2O2 is responsible for NF-κB activation in doxorubicin-treated endothelial cells and cardiomyocytes [24]. Several studies have also reported that TMP has a cell-protective function which may be mediated by ROS-scavenging activity [16,19,25]. The results of our study showed that TMP significantly reduced the gentamicin-induced ROS generation. Taken together, we suggest that inhibiting ROS generation caused by TMP, may result in decrement of gentamicin-induced NF-κB activation.

In summary, TMP can reduce the gentamicin-induced ROS formation and NF-κB activation as well as inhibit both mitochondria-mediated and death receptor-mediated apoptotic pathways. Through such mechanisms, TMP may protect rat RTC apoptosis induced by gentamicin.

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Conflict of interest statement. None declared.

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