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Original Article

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Antioxidation and anti-inflammation by haem oxygenase-1 contribute to protection by tetramethylpyrazine against gentamicin-induced apoptosis in murine renal tubular cells

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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* (called *chuanxiong* in Chinese). Besides its protection against ischaemia–reperfusion injury and nephritis in mice, we previously reported that TMP reverses gentamicin-induced apoptosis in rat kidneys. Haem oxygenase-1 (HO-1) induction by TMP has also been shown to attenuate myocardial ischaemia/reperfusion injury in rats.

Methods. We used rat renal tubular (NRK-52E) cells, transformed cells with HO-1 overexpression or knockdown, and an adenovirus carrying the HO-1 gene (Adv-HO-1) as gene therapy targeting murine kidneys to explore the role of HO-1 in protection by TMP against gentamicin-induced toxicity both *in vitro* and *in vivo*. We evaluated the protective effects of HO-1 on several apoptotic parameters induced by gentamicin: cleaved caspases-3 and -9, cycloxygenase-2 (Cox-2) and subcellular localization of nuclear factor kappa B-p65 (NF-κB-p65), Bcl-xl and HS-1-associated protein (Hax-1) in NRK-52E cells.

Results. NRK-52E cells treated with TMP exhibited transcriptional upregulation of the HO-1 protein by approximately twofold. Overexpression of HO-1 in NRK-52E cells significantly increased mitochondrial protein levels of the antiapoptotic molecules, Bcl-xL and Hax-1, and markedly decreased the NADPH oxidase activity and proinflammatory molecules, NF-κB-p65 and Cox-2, which might decrease gentamicin-induced activation of caspases-9 and -3. Conversely, NRK-52E cells with HO-1 knockdown significantly exacerbated gentamicin-induced tubular cell apoptosis. Additionally, the concomitant HO-1 induction by TMP was also evident *in vivo*, and HO-1 therapy markedly attenuated gentamicin-induced renal apoptosis to a similar extent as TMP pretreatment.

Conclusions. Collectively, we suggest that HO-1 induced by TMP might, at least in part, protect against gentamicin-induced nephrotoxicity through antiapoptotic and anti-inflammatory mechanisms, and that it may have therapeutic potential for patients with renal disease. This is also the first demonstration that HO-1 increases Hax-1 mitochondrial localization.

Keywords: apoptosis; haem oxygenase; HS-1-associated protein (HAX-1); NADPH oxidase; tetramethylpyrazine (TMP)

Introduction

Haem oxygenase (HO) is a rate-limiting enzyme in the degradation of haem to produce equimolar amounts of CO, iron and biliverdin that is further converted to the antioxidant, bilirubin, by biliverdin reductase [1,2]. Two HO isozymes have been identified as having distinct genes [3]. Among them, HO-1, a stress-response protein, can be induced by various oxidative-inducing agents, including haem, heavy metals, UV radiation, cytokines and endotoxin [4,5]. Recently, numerous *in vitro* and *in vivo* studies have shown that the induction of HO-1 is an important cellular protective mechanism against oxidative injury [3]. Several lines of evidence indicate that not only HO-1 *per se* but also its byproducts, such as CO and bilirubin, enhance the antiapoptotic function in various cells and animal models [6–8].

Gentamicin is an important and widely used aminoglycoside antibiotic for treating gram-negative bacterial infections. But nephrotoxicity is its main side effect, which seriously limits its use. Inducing apoptosis is an important cytotoxic mechanism in gentamicin-treated proximal NRK-52E and mesangial cells [9–11]. Our lab and others

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[10,12] have reported the 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 [13]. ROS generation is often responsible for the mitochondrion-mediated signalling pathway of apoptosis. Gentamicin can induce apoptosis in LLC-PK1 (Lilly Laboratories, cultured pig kidney type 1) cells through triggering the mitochondrial pathway and activating caspase-3 [12]. In mammalian cells, apoptotic stimuli cause cytochrome *c* release from mitochondria, which induces a series of biochemical reactions, resulting in activation of caspase that causes subsequent cell death [14]. 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 [15]. Inhibition of caspase-9 activation can prevent cytochrome c release from mitochondria as coordinated by pro- and antiapoptotic members of the Bcl-2 protein family. In addition, HS-1-associated protein (HAX-1) was initially identified as a 35-kDa interacting partner with HS-1, a signal-transduction protein in haematopoietic cells and located on the outer membrane of mitochondria [16]. Interestingly, HAX-1 has weak sequence homology to the proapoptotic Bcl-2 family member protein, Nip3, a protein known to be upregulated in response to oxidative stress in cardiac myocytes and which causes mitochondrial defects and apoptosis [17]. Interestingly, HAX-1 was shown to have antiapoptotic activity by binding to caspase-9, thus interfering with its activation [18].

Tetramethylpyrazine (TMP, also called ligustrazine) is purified from the rhizome of *Ligusticum wallichi* (called *chuanxiong* in Chinese). It is a widely used active ingredient in Chinese herbal medicines to treat coronary artery and ischaemic cerebral vascular diseases [19,20]. Besides vasodilatory actions and antiplatelet activity, TMP has strong effects in removing oxygen-free radicals, the underlying mechanisms of which are associated with increased activities of superoxide dismutase (SOD), catalase and glutathione peroxidase [21,22]. It has also been documented to protect against ischaemia–reperfusion injury, nephritis and alcohol-induced toxicity in rat kidneys [22–24] and acute econazole-induced liver injury [25]. Our group recently reported that TMP effectively decreased ROS formation induced by gentamicin and attenuated apoptotic injury by inactivating caspases-3, -8 and -9 to inhibit gentamicin-induced release of cytochrome c, as well as increasing the expression of Bcl-xL [26]. Those findings indicate that TMP can protect against and can possibly be used to treat renal diseases. Additionally, a very recent report indicated that HO-1 induction by TMP is ascribable to attenuation of myocardial ischaemia/reperfusion injury in rats [27]. Additionally, increases in both tumour necrosis factor (TNF)-α and nitrate levels induced by lipopolysaccharide (LPS) were significantly reduced in LPS-administered rats pretreated with TMP [28], and our publication also showed that gentamicin-induced TNF-α release and NF-κB-p65 transcriptional activation in NRK-52E cells were inhibited by TMP treatment [26]. Herein, we attempted to unravel the protective effects of TMP associated with HO-1 induction in preventing gentamicin-induced apoptosis both *in vitro* and *in vivo*.

Materials and methods

Reagents

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

Cell culture

We purchased rat proximal tubular (NRK-52E) cells from the Bioresource Collection and Research Center (Hsinchu, Taiwan), and cultured them in DMEM supplemented with an antibiotic/antifungal solution and 10% FBS (pH 7.2). They were grown until the monolayer became confluent. The medium for the cultured cells was then changed to serum-free medium, and cells were incubated overnight before the experiment.

Constructs of plasmid variants and the luciferase activity assay

The method to obtain the pGL3/h*HO-1* reporter plasmid, which contains a 3293-bp fragment located −3106 to +186 relative to the transcription start site of the human *HO-1* gene, and the method for the reporter activity assay were described previously [29].

Western blot analysis

We applied 50 μg of NRK-52E lysate proteins to each lane and analysed them with western blots. We purchased the antibodies for HO-1, caspase-3, caspase-9, Bcl-xL, NF-κB-p65, Hax-1 and Cox-2 from BD Laboratories (San Jose, CA, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA) and diluted them 1:1000 for the assay. We used peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin G (IgG) (1:5000 dilution) as the second antibody to detect the above-mentioned protein bands by enhanced chemiluminescence (Pierce, Rockford, IL, USA).

Determination of bilirubin in culture medium

NRK-52E cells were plated with a density of 3×10^5 cells/well into 24-well plates and treated with the indicated compounds for 12 h. The supernatant (0.5 mL) was collected, and 250 mg $BaCl_2$-$2H_2O$ was added. After vortexing, 0.75 mL benzene was added and mixed well again. The benzene phase containing bilirubin was separated by centrifugation for 30 min at 13 000 × *g*. The extraction of bilirubin was determined as a difference in absorbance between 450 and 600 nm (ε450: 27.3 mM^{-1} cm^{-1}) [30,31].

Establishment of HO-1 transfectants

pcDNA-HO-1, a constitutive expression vector, carries full-length human HO-1 complementary (c)DNA under the control of the CMV promoter/enhancer sequence. A short

hairpin (sh)RNA against rat HO-1 was generated in the pSM2 vector (Open Biosystems, Huntsville, AL, USA) and amplified in an *Escherichia coli* system. Confirmation was verified by restriction site analysis and sequencing. We transfected pcDNA-HO-1, pcDNA or pSM2-shHO-1 (4 μg/3.5 cm Petri dish) into NRK-52E cells using the jetPEI™ (Polyplus-transfection, San Marcos, CA, USA). After transfection, cells were plated in DMEM with 10% FBS and 400 μg/mL of G418 for pcDNA variants, or 2 μg/mL of puromycin for pSM2-shHO-1 as selective pressures. G418- or puromycin-resistant cells were selected and expanded. The level of HO-1 was analysed by western blotting.

NADPH oxidase activity

NADPH oxidase was measured as previously described [30]. In brief, NRK-52E cells were scraped into ice-cold PBS containing 1 mmol/L EGTA and centrifuged for 10 min at 750 × *g* and 4°C. The pellet was resuspended in a lysis buffer (20 mmol/L potassium phosphate, 1 mmol/L EGTA, 10 mmol/L aprotinin, 0.5 mmol/L phenylmethylsulfonyl fluoride and 0.5 mmol/L leupeptin) and sonicated. The protein concentration was adjusted to 2 mg/mL. A total cell suspension with a volume of 250 μL was mixed with 250 μL of HBSS containing 500 μM lucigenin and kept at 37°C for 10 min. The NADPH oxidase activity assay was initiated by adding 10 μL of NADPH (100 μmol/L) as the substrate. The photon emission was measured, and the respective background counts were subtracted. Neither the cellular fraction alone nor NADPH alone evoked any lucigenin chemiluminescent signal [32,33].

Nitroblue tetrazolium (NBT)

Cells were plated in 24-well plates and challenged with 3 mM of gentamicin for 8 h. Cells were washed in PBS and incubated with NBT (2 mg/mL) in PBS at 37°C for 60 min. After fixation in 100% methanol and a wash in methanol, the formazan precipitates were dissolved by the addition of 230 μL 2M KOH and 280 μL DMSO. The amount of reduced NBT was quantified by determination of the absorbance at 620 nm.

Preparation of an adenovirus (Adv) construct containing HO-1

A recombinant Adv was constructed by a method described elsewhere [34]. Human HO-1 cDNA containing the entire coding sequence was subcloned into the Adv shuttle plasmid vector, pAdv-CMV, which contains a cytomegalovirus (CMV) promoter and a polyadenylation signal from bovine growth hormone. The recombinant adenovirus was generated by homologous recombination and amplified in 293 cells as previously described [34]. Virus titres were determined by a plaque assay on a 293-cell monolayer.

Animals and treatments

Animal care and treatment were conducted in conformity with the protocols of the Animal Center, Taipei Medical University. Male C57B6 mice (6- to 10-week old, 25.8 ± 1.8 g) were used in this study. Animals were anaesthetized intramuscularly with a combination of ketamine (8 mg/100 g body weight), xylazine (2 mg/100 g) and atropine (0.16 mg/100 g). Before and during treatment, we housed animals in a central facility, 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 an intraperitoneal (IP) injection of gentamicin (20 mg/kg/day) for 7 days. The test-group animals ($n = 6$) for gentamicin and TMP treatment received an IP injection of TMP (80 mg/kg/day) 30 min before the gentamicin treatment, or for gentamicin and Adv-HO-1 gene therapy were intravenously given Adv-HO-1 (5.0×10^9 pfu) in 50 μL of sterilized PBS 3 days prior to gentamicin challenge. Control group mice were intravenously given an empty Adv (5.0×10^9 pfu) 3 days prior to receiving a continuous infusion of 0.9% (w/v) saline throughout the gentamicin treatment period.

Histological and renal function evaluations, TUNEL assay and renal MPO activity

Animals were killed after gentamicin treatment for 7 days. Kidneys from the mice were excised, fixed in 4% paraformaldehyde, embedded in paraffin and sectioned at 7 μm for histological staining with haematoxylin and eosin and TUNEL staining. Blood urea nitrogen was measured using a commercially available kit (Sigma). Apoptosis in renal tissues was identified by a TUNEL assay with an *in situ* Cell Death Detection kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. Five fields per section and two sections per kidney were examined in each experimental group. MPO, indicating neutrophil infiltration into tissue, was measured as previously described [35].

Statistical analysis

Values are expressed as the mean ± SD of at least three experiments. The significance of the difference from the control groups was analysed by Student's *t*-test or one-way analysis of variance (ANOVA). A value of $P < 0.05$ was considered statistically significant.

Results

The molecular actions of TMP against gentamicin-induced apoptosis are examined in Figure 1, which shows that gentamicin decreased Bcl-xL and increased the activated form of caspase-3. In addition, proinflammatory proteins, including levels of NF-κB-p65 and Cox-2, increased with gentamicin challenge to approximately twofold, whereas the adverse effects of gentamicin were significantly reversed by TMP treatment.

We further investigated the protection by TMP associated with HO-1 induction against gentamicin-induced apoptosis in NRK-52E cells. As shown in Figure 2A, HO-1 promoter-driven luciferase activity increased in response to the increased concentrations of TMP of 50–100 μM.

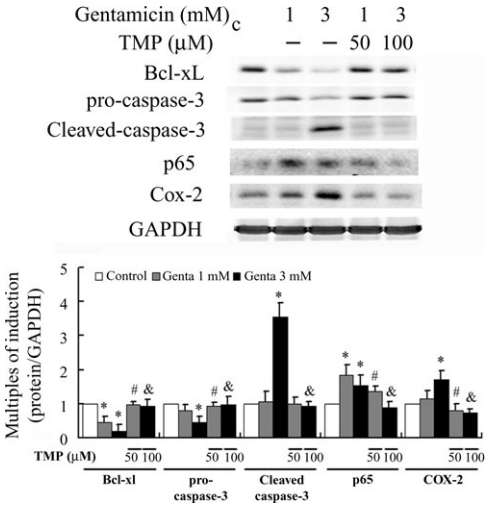

Fig. 1. The protective effect of tetramethylpyrazine (TMP) on the cytotoxicity of gentamicin in NRK-52E cells. Cells were pretreated with TMP (50 and 100 μM) for 30 min, and then treated with 3 mM of gentamicin for 6 h for the western blot analysis of p65, Cox-2 and Bcl-xL and for 24 h for analysis of cleaved caspase-3. Results are expressed as mean ± SD ($n = 3$). $^{\#}P < 0.05$, compared to the gentamicin alone group; C = untreated control. GAPDH was used as an internal control. A representative experiment is shown. Bar charts in the lower part of panel B show the band intensities of normalized indicated proteins in terms of GAPDH by densitometry (IS-1000 Digital Imaging System). Data were derived from three independent experiments and are presented as the mean ± SD. $^{*}P < 0.05$ indicates a significant difference from the PBS-treated group. $^{\#}P < 0.05$ and $^{\&}P < 0.05$ versus the control group treated with 1 and 3 mM of gentamicin, respectively.

Additionally, the concentration- and time-dependent induction of HO-1 by TMP in NRK-52E cells was analysed at both the mRNA and protein levels, as harvested from NRK-52E cells treated with 1–100 μM of TMP for 12 h and with 100 μM of TMP for various time points of 4–24 h. The RNA induction of HO-1 was observed with 10–100 μM and at 8–12 h of TMP treatment, respectively (Figure 2B, C). Western blot analysis showed that TMP at the concentrations of 10 and 100 μM for 12 h significantly induced HO-1 expression in a concentration-dependent fashion (Figure 2B). Furthermore, consistent with the time frames of mRNA upregulation by the RT-PCR analysis, a western blot analysis demonstrated that the induction of HO-1 by TMP was apparent at 8 h and was sustained for up to 24 h, the longest time point examined (Figure 2C). Additionally, activation of the HO-1 enzyme converts haem to bilirubin, an indicator of HO activity. In order to identify if activation of HO-1 enzyme induced activity in TMP-treated cells, production of bilirubin in the medium was measured by a $BaCl_2$ extraction method as described in the Materials and methods section. Results in Figure 2D show that TMP concentration-dependently induced bilirubin production to ~2.5-fold in NRK-52E cells.

We demonstrated that TMP time and concentration-dependently induced HO-1 overexpression. To examine whether the antiapoptotic effects of TMP by gentamicin might occur via the induction of HO-1, NRK-52E cells were genetically transformed into HO-1-overexpressing cells or conversely with HO-1 knockdown. As expected and shown in Figure 3A, NRK-52E cells transfected with pcDNA-HO-1 overexpressed HO-1, and conversely the

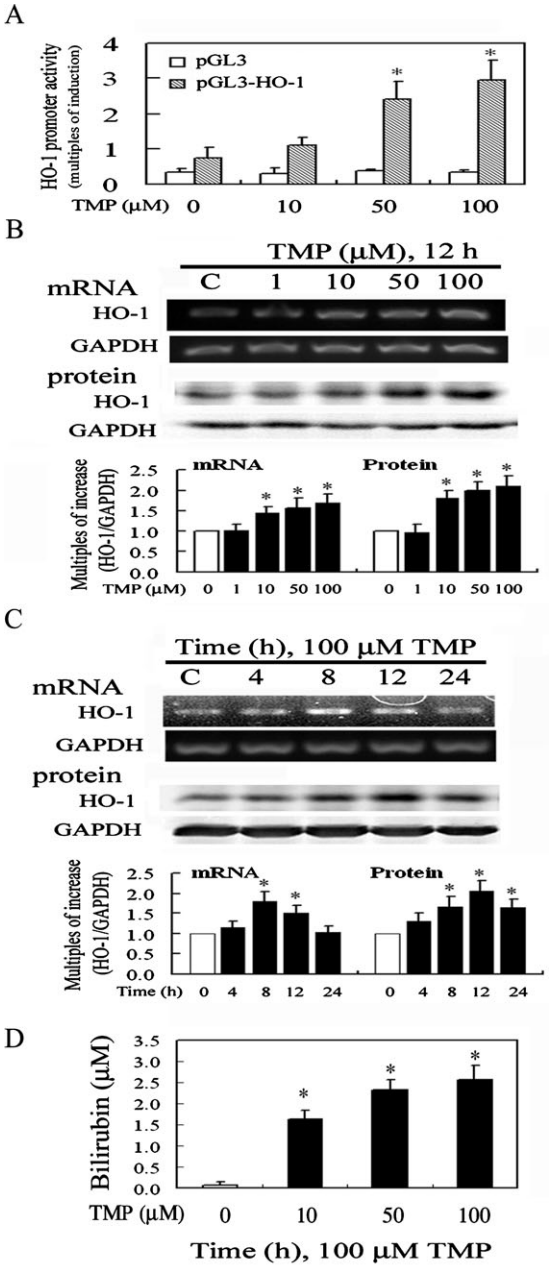

Fig. 2. Transcription regulation of haem oxygenase (HO)-1 by tetramethylpyrazine (TMP) in concentration- and time-dependent fashions. (**A**) HO-1 promoter activity in relation to increasing concentrations of TMP. NRK-52E cells were transiently transfected with pGL3/h*HO-1* and prRL-TK for 48 h, followed by treatment with increasing concentrations of TMP. Luciferase activities of the reported plasmid were normalized to those of the internal control plasmid and are presented as the mean ± SD of four independent experiments. (**B**) Concentration-dependent induction of HO-1 protein by TMP. Cells were treated with the indicated concentrations of TMP for 12 h, and the expression of HO-1 was determined using GAPDH as an internal control. (**C**) Time-course induction of HO-1 by TMP. Cells were treated with TMP (100 μM) for 4, 8, 12 and 24 h, and the expression of the HO-1 protein in the cells was analysed by western blotting. Bar charts in the lower parts of panels B and C show the band intensities of normalized HO-1 in terms of GAPDH by densitometry. Results of three experiments are shown. (**D**) NRK-52E cells were treated as described in Figure 2B and the amount of bilirubin in the medium was measured as described in the Materials and methods section. Data were obtained from six independent experiments and are expressed as the mean ± SD. $^{*}P < 0.05$ versus the PBS-treated group, $^{\#}P < 0.05$ versus the gentamicin-treated group.

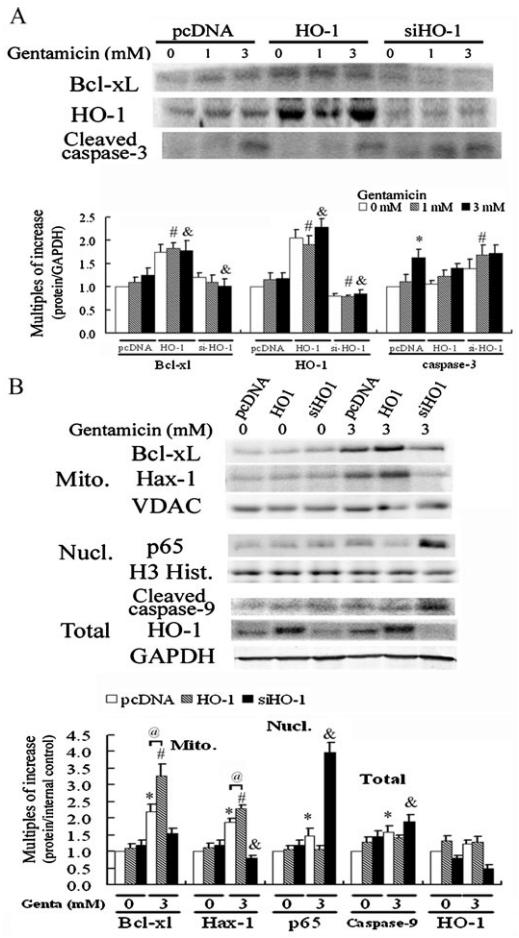

Fig. 3. Haem oxygenase (HO)-1 overexpression in NRK-52E cells mimicking the effects of tetramethylpyrazine (TMP) treatment of reversing gentamicin-induced toxicity. (**A**) Western blot analysis of pro- or antiapoptotic and proinflammatory proteins in response to gentamicin challenge in cells transformed with HO-1 overexpression or HO-1 knockdown. Cells were transfected with pcDNA alone as an internal control, pcDNA-HO-1 or with HO-1 knockdown as described in the Material and methods section. Cells were treated with the indicated concentrations of gentamicin and harvested at 6 h for the analysis of Bcl-xL or at 24 h for analysis of cleaved caspase-3. (**B**) Effects of HO-1 on the subcellular localization of antiapoptotic and proinflammatory molecules. Cells were pretreated as described in Figure 3A except that cells were partitioned into cytosol, nuclear and mitochondrial factions. Bar charts in the lower parts of panels A and B show the band intensities of normalized indicated proteins, and the voltage-dependent anion channel (VDAC), H3 histone and GAPDH were used as internal control proteins for the fractions of mitochondria, nuclei and whole cells, respectively. Data were derived from three independent experiments and are presented as the mean ± SD. $^{*}P < 0.05$ indicates a significant difference from the PBS-treated group in cells transformed with pcDNA. $^{\#}P < 0.05$ and $^{\&}P < 0.05$ versus cells transformed with pcDNA with the addition of 1 and 3 mM gentamicin treatment, respectively. $^{@}P < 0.05$ versus the results of the pcDNA group challenged with 3 mM gentamicin.

HO-1 protein level decreased in cells knocked-down with HO-1 compared to control cells. Likewise, gentamicin concentration-dependently increased the active form of caspase-3 in these cell variants. HO-1 protein levels were positively proportional to the amounts of the Bcl-xL protein, but negatively correlated with cleaved caspase-3. Additionally, cells with HO-1 knockdown showed a greater susceptibility to eliciting caspase-3 activation at a concentration as low as 1 mM of gentamicin.

To functionally assess the active subcellular localization of antiapoptotic and proinflammatory molecules such as Bcl-xL, Hax-1 and NF-κB-p65, cell variants treated with or without 3 mM of gentamicin were partitioned into mitochondrial and nuclear fractions. As illustrated in Figure 3B, control cells and those with HO-1 induction showed marked increases in both Bcl-xL and Hax-1 protein levels in the mitochondrial fractions, whereas these phenomena were not apparent in cells knocked down with HO-1. Furthermore, cells overexpressing HO-1 showed a greater extent of increase in the mitochondrial localization of those antiapoptotic proteins compared to the control group. The membrane blot was also analysed for caspase-9 activation using an anti-cleaved caspase-9 antibody, since Hax-1 has been shown to prevent caspase-9 activation by apoptotic signalling [27]. As presented in Figure 3B, NRK-52E cells overexpressing HO-1 showed a decrease in caspase-9 activation, which was correlated with the increased mitochondrial translocations of Hax-1 and Bcl-xL. Furthermore, the nuclear localization and activation of NF-κB-p65, a redox-sensitive proinflammatory transcription factor composed of p65 and p50, were analysed. NRK-52E cells overexpressing HO-1 showed a significant decrease in gentamicin-induced p65 nuclear translocation compared to the control group, whereas cells with HO-1 knockdown showed an approximately threefold increase in the nuclear fraction of p65 induced by gentamicin. However, no apparent alteration of p50 in the cytosolic or nuclear fractions of cell variants was evident upon gentamicin challenge (data not shown).

We next examined whether the elimination of gentamicin-induced ROS formation by TMP might be due to TMP-mediated HO-1 induction. As shown in Figure 4, HO-1 overexpression significantly inhibited gentamicin-induced NADPH oxidase activity (Figure 4A) and ROS generation (Figure 4B) to extents similar to those with TMP treatment. Interestingly, additional TMP pretreatment in cells knocked-down with HO-1 exhibited no further reduction in gentamicin-induced NADPH oxidase activity or ROS generation according to the NBT assay, suggesting the importance of HO-1 in the protective effect of TMP.

The protective effect of HO-1 on gentamicin-induced apoptosis was further proven in a murine animal model. As shown in Figure 5A, 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 of gentamicin-treated animals, but rarely in specimens of the control or gentamicin-treated animals. Most of the TUNEL-labelled nuclei were seen in proximal renal tubular cells. This result demonstrated that mice receiving Adv-HO-1 showed inhibited gentamicin-induced cell apoptosis in kidneys by about ∼75% compared to gentamicin insult determined by the TUNEL assay, which was similar to that of the group with TMP pretreatment. Furthermore, renal cells pretreated with Adv-HO-1 and TMP showed profound induction of HO-1 compared to control animals that received an empty Adv alone and gentamicin-treated animals. Mice challenged with gentamicin showed substantial histological changes, such as severe tubular apoptosis and slight necrosis, tubular dilation and protein casts. In contrast, most of the tubular structure and integrity were maintained, and histological damage was milder

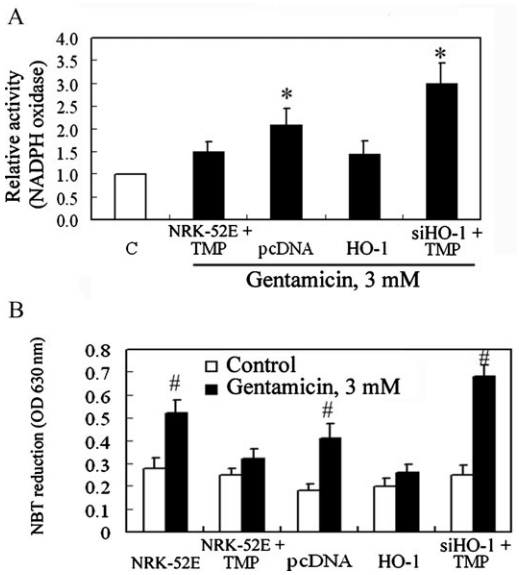

Fig. 4. Haem oxygenase (HO)-1 responsible for the protective effects of tetramethylpyrazine (TMP) against gentamicin-induced free radical production in NRK-52E cells. Effects of HO-1 on gentamicin-induced NADPH oxidase activity (**A**) and on gentamicin-induced reactive oxygen species (ROS) generation (**B**). NRK-52E cells were permanently transformed with HO-1 overexpression or knockdown followed by an additional challenge with 3 mM gentamicin for 1 h with or without 100 μM TMP pretreatment for 8 h. Gentamicin-induced increases in NADPH oxidase activity and intracellular ROS were detected by a luminometer and ELISA reader at a wavelength of 620 nm. Data were normalized to each corresponding cell variant without gentamicin insult and are presented as the mean ± SD of four independent experiments. $^{*}P < 0.05$ versus the control group and $^{\#}P < 0.05$ versus each respective control group.

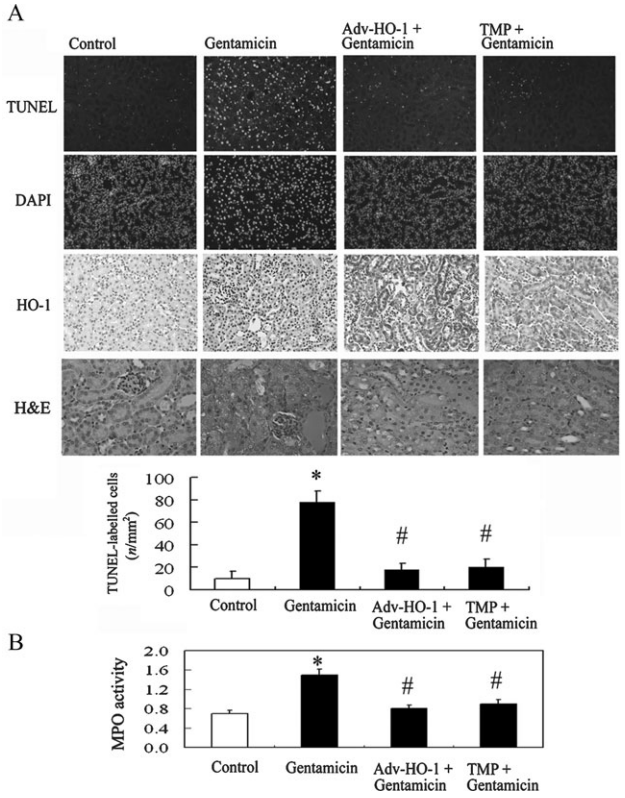

Fig. 5. Reversal of gentamicin-induced apoptosis, histological alteration (**A**) and inflammation (neutrophil infiltration) (**B**) by haem oxygenase (HO)-1 gene therapy, similar to the protective effects of tetramethylpyrazine (TMP) treatment. Mice were divided into four groups: one with an empty adenovirus (Adv) alone, Adv alone with additional gentamicin (20 mg/kg/day) and gentamicin in combination with Adv-HO-1 or with TMP (80 mg/kg/day). Mice were intravenously injected with an Adv-HO-1 gene (5.0×10^9 pfu) in 50 μL of sterilized PBS for 3 days before gentamicin challenge. (**A**) Kidneys were dissected and sectioned for TUNEL, immunohistochemical staining for HO-1 and histological evaluation. Apoptotic cells in kidneys of experimental animals were detected using *in vivo* TUNEL staining. Top line: TUNEL-labelled nuclei were revealed as bright spots in cortical sections from untreated and treated mice. Second line: The identical fields stained for TUNEL were also stained using DAPI to reveal the positions of cell nuclei. Third line: The extents of TMP-induced HO-1 and HO-1 overexpression by Adv-HO-1 infection were examined by immunostaining with an anti-HO-1 antibody. The numbers of TUNEL-labelled cells per square millimetre of cortex area in each sample were compiled and demonstrated. Bottom line: Representative photomicrographs of haematoxylin and eosin staining were shown. Micrographs of representative fields were recorded. (**B**) MPO activity in renal tissue samples obtained from mice with various treatments. MPO activity is expressed as ΔOD460/min/mg protein. Results are expressed as the mean ± SD ($n = 6$). $^{*}P < 0.05$ versus the given empty Adv alone and $^{\#}P < 0.05$ versus the empty Adv with the addition of gentamicin challenge. Magnification ×200.

in mice additionally treated with TMP and Adv-HO-1 compared to gentamicin alone. Semiquantitative assessment of histologic injury yielded tubular necrosis scores of 0.05 ± 0.01 in Adv-treated mice, 1.1 ± 0.2 in gentamicin-treated mice and 0.4 ± 0.1 and 0.2 ± 0.1 in mice treated with gentamicin and TMP or Adv-HO-1, respectively (semiquantitative scores; 0 = normal; 1 ≤ 10%; 2 = 10–25%; 3 = 26–75%; 4 ≥ 75% cells exhibiting necrosis). Results in Figure 5B demonstrate the gentamicin-induced neutrophil infiltration in NRK-52E cells by measuring MPO activity, an abundant constituent of neutrophils, which was significantly reversed with TMP and Adv-HO-1 pretreatment. Furthermore, in the renal functional assessment shown in Figure 6, mice subjected to gentamicin insult showed increased serum levels of urea and creatinine, which suggests renal dysfunction; however, levels of serum urea and creatinine in mice additionally treated with TMP and Adv-HO-1 were significantly lower than those observed in the gentamicin-alone group, which suggests a marked prevention of renal failure associated with gentamicin toxicity.

Discussion

Our group previously demonstrated that TMP possesses free-radical scavenging and anti-inflammatory effects and protects NRK-52E cells from gentamicin-induced apoptosis [12]. In this study, we identified an essential molecule, HO-1, induced by TMP, which might contribute to the protective actions of TMP against gentamicin-induced apoptosis. The results of our study showed that TMP-induced HO-1 expression occurred in concentration- and time-dependent manners. Transcriptional regulation is essential for the induction of HO-1, as the promoter activity of HO-1 increased in response to TMP treatment (Figure 3). NRK-52E cells with HO-1 knockdown exacerbated the gentamicin-induced cleaved form of caspase-3 by

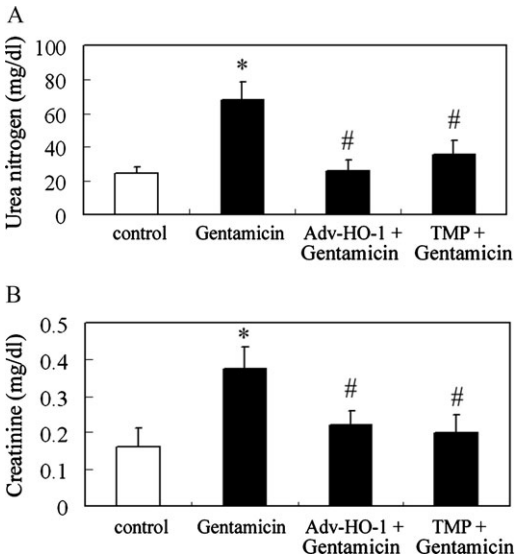

Fig. 6. Renal damage induced by gentamicin was reversed by pretreatment of TMP and Adv-HO-1. Concentrations of blood urea nitrogen (**A**) and serum creatinine (**B**) in the aforementioned four groups were measured at the end of the 7-day gentamicin treatment. Results are the mean ± SD ($n = 6$). $^{*}P < 0.05$ versus the control group. $^{\#}P < 0.05$ versus the gentamicin-treated group.

decreasing the mitochondrial localization of the antiapoptotic molecules, Bcl-xL and Hax-1, accompanied by an increase in the nuclear localization of the proinflammatory molecule, NF-κB-p65. Similar to the therapeutic effects of TMP, HO-1 overexpression provided protection against gentamicin-induced injury both *in vitro* and *in vivo*.

Given that TMP induces HO-1, it is tempting to postulate that the antioxidant and anti-inflammatory properties of TMP may, at least in part, be related to the induction of HO-1. Recently, numerous *in vitro* and *in vivo* studies have shown that the induction of HO-1 is an important cellular protective mechanism against oxidative injury [3,36–39]. Its potential use for therapeutic targets in various diseases has been explored. For instance, overexpression of HO-1 by pharmacological induction or adenovirus-mediated gene transfer of HO-1 protects kidneys from angiotensin II-induced oxidative stress, apoptosis and renal dysfunction.

The results presented in this study showed that TMP might inhibit gentamicin-induced NADPH oxidase activity through the antioxidative actions of HO-1 since TMP no longer scavenged ROS in NRK-52E cells with HO-1 knockdown (Figure 4B). The *in vitro* findings complement the reported inhibitory actions of HO-1 on NADPH oxidase function in macrophages and in human endothelial cells [40]. The mechanisms by which HO-1 modulates NADPH oxidase activity are not totally clear. Confounding results exist in the literature. For example, HO-1's induction by haemin did not alter the protein levels of the Nox subunits in either the kidney or aorta [40], which is in contrast to findings of Taille *et al*. in macrophages [7]. Determining the underlying molecular mechanisms ascribable to the inhibitory effects of HO-1, overexpression in NRK-52E cells on NADPH oxidase function requires further investigation.

Recent studies have revealed that H_2O_2 is responsible for NF-κB activation in doxorubicin-treated endothelial cells and cardiomyocytes [21]. Several studies have also reported that TMP has a cell-protective function, which may be mediated by its ROS-scavenging activity [22,25]. The results of our study showed that HO-1 induced by TMP significantly reduced gentamicin-induced NADPH oxidase activity and then ROS generation. Taken together, we suggest that inhibiting ROS generation by HO-1 may result in a decrease in gentamicin-induced NF-κB activation. Nevertheless, whether the suppression of NF-κB-p65 cells decreases Cox-2 induction by HO-1 (Figure 3B) requires further investigation.

Furthermore, the results presented in this study showed that HO-1 overexpression not only mimicked the effects of TMP in increasing Bcl-xL mitochondrial localization but also induced HAX-1 in the mitochondrial fraction that has not previously been reported. Hax-1 has been demonstrated to bind to caspase-9 and inhibit the activation of caspases-9 and -3. Herein, the outcome of increased Hax-1 in mitochondria in NRK-52E cells overexpressing HO-1 is assessed in Figure 4B that shows a decrease in the active form of caspase-9, as previously reported. Nevertheless, how HO-1 increases Hax-1 mitochondrial localization and what is its underlying signalling pathway remain to be elucidated.

The results presented in this study provide evidence of the protective effects of HO-1 against gentamicin-induced apoptosis through its antioxidative and anti-inflammatory properties. These effects might be ascribable to its byproducts, bilirubin and CO [8]. The biological actions of bilirubin have been shown to scavenge ROS *in vitro* [41] and inhibit NADPH oxidase [6], thereby reducing oxidant-mediated cellular damage and attenuating oxidant stress *in vivo* [42]. Furthermore, CO has been demonstrated to significantly contribute to the anti-inflammatory properties of HO-1 by suppressing inflammatory cytokines through activation of both sGC and p38 MAPK [37,43].

To validate the physiological concentration of gentamicin for the treatment of gram-negative bacterial infection, we used 1–3 mM of gentamicin to induce apoptosis in NRK-52E cells with 24 h of treatment. These concentrations were shown to induce apoptosis in LLC-PK1 and MDCK (Madin-Darby Canine Kidney) cells, and approximate levels of the drug that are reached in the kidneys *in vivo* during treatment [9,12,44,45]. As explained by El Mouedden *et al*. [9,44], this concentration range allows LLC-PK1 cells to obtain cellular and intralysosomal drug concentrations similar to those observed in proximal tubular cells of rats receiving clinically relevant doses of gentamicin with apoptosis. Adv-HO-1 administration in preventing gentamicin-induced murine tubular apoptosis was assessed for its systemic effect *in vivo*. As shown in Figure 5, the administration of Adv-HO-1 resulted in high expression of HO-1 in the kidneys and lowered the amount of positive cells stained by TUNEL to an extent similar to that with TMP pretreatment. In contrast, mice receiving the empty adenovirus with a lower level of HO-1 induction showed more TUNEL-positive cells following gentamicin challenge. We also showed that renal dysfunction after 7 days of gentamicin insult, as reflected by elevated serum creatinine and urea levels, was markedly prevented

by TMP and Adv-HO-1 pretreatment compared to gentamicin challenge alone. This protection was accompanied by a corresponding reduction in renal tubular cell apoptosis, necrosis and inflammatory responses. Nevertheless, whether the anti-inflammatory effects of TMP and HO-1 reversed gentamicin-induced tubular necrosis remains for further investigation.

Therefore, we concluded that the genetic suppression of HO-1 exacerbates gentamicin-induced apoptosis in NRK-52E cells, whereas HO-1 overexpression, similar to TMP pretreatment, reverses the detrimental effects of gentamicin. Interestingly, the results of our study are in accord with a recent study showing that genetic suppression of HO-1 exacerbates ischaemia-induced renal damage [46]. We suggest that TMP inhibits apoptotic signalling pathways to protect gentamicin-treated NRK-52E cells and murine kidneys through an HO-1-dependent pathway, and that increased mitochondrial localization of Hax-1 might present a new mechanism of HO-1-mediated cytoprotection. TMP might be a potential dietary component for protecting cells and tissues against oxidative injuries. Further studies using TMP will clarify the possibility of developing this new 'drug' for the prevention or treatment of renal tubular apoptosis disease.

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Conflict of interest statement. None declared.

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