

Original Article

PEA3 protects against gentamicin nephrotoxicity: role of mitochondrial dysfunction

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Abstract: Toxin-induced nephrotoxicity is one of the major causes leading to the acute kidney injury (AKI). Among these nephrotoxic toxins, gentamicin can induce AKI with elusive mechanisms. Emerging evidence demonstrated that PEA3 (polyomavirus enhancer activator 3) contributed to the nephrogenesis, while its role in AKI remains unknown. Thus, this study was to investigate the role of PEA3 in gentamicin nephrotoxicity, as well as the underlying mechanisms. In rats, gentamicin treatment (200 mg/kg twice per day) for two days induced remarkable kidney injury with a peak damage on day 5 evaluated by the tubular injury score, proteinuria, and tubular injury markers of NGAL and KIM-1. In parallel with the tubular injury, PEA3 protein and mRNA expressions were significantly upregulated by gentamicin and peaked on day 5. To define the role of PEA3 in gentamicin nephrotoxicity, proximal tubule cells were transfected with PEA3 plasmid with or without gentamicin treatment (1 mg/ml). Notably, overexpression of PEA3 attenuated gentamicin-induced cell injury shown by the ameliorated cell apoptosis and NGAL and KIM-1 upregulation. Meantime, gentamicin caused severe mitochondrial dysfunction, which was largely normalized by PEA3 overexpression. In contrast, silencing PEA3 by a siRNA strategy further deteriorated gentamicin-induced cell apoptosis and mitochondrial dysfunction. In sum, PEA3 protected against gentamicin nephrotoxicity possibly via a mitochondrial mechanism.

Keywords: AKI, gentamicin, PEA3, mitochondria dysfunction

Introduction

Acute kidney injury (AKI) is a common clinical complication with high mortality and morbidity. The epidemiological data indicated that the incidence rate of CKD after an episode of AKI was 7.8 per 100 patient-years, and the rate of ESRD was 4.9 per 100 patient-years [1, 2]. One of the major causes of AKI is toxin-induced nephrotoxicity [3]. Gentamicin, an important aminoglycoside antibiotic, is widely used in clinic for its potent antibiotic activity against the various gram-negative microorganisms. However, its use was greatly limited by the nephrotoxicity, accounting for 10-15% of all cases of acute renal failure [4]. The studies related to gentamicin toxicity in kidney only revealed some non-specific contributors like oxidative stress and inflammation [5]. For example, gentamicin could enhance the production of reactive oxygen species (ROS) such as hydroxyl radicals, superoxide anions, hydrogen peroxide and

reactive nitrogen species [6]. Thus, the detailed molecular mechanisms of gentamicin nephrotoxicity are still elusive.

PEA3 (also called ETV4) belongs to the *pea3* subfamily of the multigene ETS domain containing a class of transcription factors, and was firstly identified in the genome of avian retrovirus E26 [7]. PEA3 has been known as an important contributor of the development of neuron, limb, kidney, and other organs [8]. During the nephrogenesis, PEA3, cooperating with *Etv5* (also called *Erm*, another member of the *Pea3* subfamily), is positively regulated by GDNF/Ret signaling, which is required for the ureteric bud branching morphogenesis. Mice lacking *Etv4* (PEA3) alleles and one *Etv5* allele presented a high frequency of renal agenesis or hypodysplasia, due to the branching defects [9].

Since it has been reported that epithelial repair following acute injury requires transient re-

PEA3 and gentamicin nephrotoxicity

Table 1. Primers of qRT-PCR analysis

Gene symbol	Accession number	Forward primer (5'-3')	Reverse primer (5'-3')
rPEA3	NM_001108299	GGCCACGGGTACCTTGGT	AGATGTGAAGGCATGGCACAT
rKIM-1	AF035963	CCGCAGAGAAACCCGACTAA	AACAAAGCTCAGAGAGCCCA
rNGAL	NM_130741	TCTGGGCCTCAAGGATAACAAC	AGACAGGTGGGACCTGACCA
rGAPDH	NM_017008	CAAGTTCACGGCACAGTCAA	TGGTGAAGACGCCAGTAGACTC
mPEA3	NM_008815	CGCACAGACTTCGCCTACG	CAGACATCATCTGGGAATGGTC
mKIM-1	NM_001166632	ACATATCGTGGAATCACAACGAC	ACTGCTCTTCTGATAGGTGACA
mNGAL	NM_008491	TGGCCCTGAGTGTGATGTG	CTCTTGAGCTCATAGATGGTGC
mGAPDH	NM_008084	ATGGTGAAGGTCGGGTGAA	TGGAAGATGGTATGGGCTT
mtDNA	NC_005089	ATCCTCCAGGATTTGGAAT	ATCCTCCAGGATTTGGAAT
18S rRNA	X00686	TTCGGAAGTGGCCATGATT	TTTCGCTCTGGTCCGCTCTG

expression of genes normally expressed during kidney development and the activation of growth factors and cytokine-induced signaling pathways [10]. Many genes and pathways participating in the nephrogenesis contributed to the repair after renal injury [11-13]. Thus, we speculated that PEA3 as a transcription factor associated with nephrogenesis might play an important role in the pathogenesis of AKI.

In this study, the regulation of PEA3 was observed in the kidneys of gentamicin-treated rats and renal tubular cells. The roles of PEA3 in gentamicin-induced renal tubular injury and mitochondrial dysfunction were evaluated by genetic approaches *in vitro*. The results from current study suggested that PEA3 might be a valuable target in protecting against gentamicin nephrotoxicity and mitochondrial dysfunction.

Materials and methods

Materials

Gentamicin sulfate was from Sigma (St. Louis, MO). Lipofectamine TM 2000 was obtained from Invitrogen. Anti-PEA3 polyclonal (H120) antibody was purchased from Santa Cruz Biotechnology. FITC-conjugated anti-rabbit secondary antibody was provided by KANGCHEN BIO-TECH. Cell culture reagents were obtained from Corning and Gibco BRL.

Animals

Male Sprague-Dawley (SD) rats weighing 200±20 g were used in the study. The animals were maintained under standard housing conditions (25°C room temperature and 12 h light/

dark cycle) and were supplied with standard rodent chow and tap water *ad libitum*. All animal procedures were approved by the Nanjing Medical University Institutional Animal Care and Use Committee.

Experimental design

Forty male SD rats were randomly divided into 5 groups (day 0, day 5, day 9, day 13, and day 29). They were subcutaneously injected with gentamicin at a dose of 200 mg/kg or vehicle twice per day for two consecutive days. Then the animals were killed on day 0, day 5, day 9, day 13, and day 29, respectively. Before killing the animals, 24 h urine was collected and 24 h urinary protein level was detected with Pierce BCA protein assay kit (ThermoFisher Scientific™, Waltham, MA USA) according to the manufacturer's instructions. A longitudinal section from the left kidney was excised for histological examination. The rest of kidneys were stored at -80°C for other analyses.

Histological analysis

Kidneys from rats of all the groups were fixed in 4% paraformaldehyde/PBS solution and embedded in paraffin. Sections (3 µm) were stained with PAS or H&E and analyzed by a pathologist in a blind procedure. A minimum of 10 fields for each kidney slide were examined and scored for pathological injury [14]. A score from 0 to 4 was given for pathological assessment: 0, normal histology; 1, mild injury, 5% to 25% of tubules showed pathological damage; 2, moderate injury, 25% to 50% of tubules showed pathological damage; 3, severe injury, 50% to 75% showed pathological damage; and 4, almost all tubules in field of view were dam-

PEA3 and gentamicin nephrotoxicity

aged. The average histological score for each sample was calculated.

RNA isolation and quantitative real-time PCR (qRT-PCR) analysis

Total DNA and RNA from cultured MPTCs and kidneys were extracted by a DNeasy Tissue Kit (QIAGEN Sciences, Germantown, MD) and Trizol reagent (Invitrogen, Carlsbad, CA), respectively. Oligonucleotides (listed in **Table 1**) were designed by Primer3 software (<http://frodo.wi.mit.edu>) and synthesized by Invitrogen. qRT-PCR was performed for the detection of target gene expression and mtDNA copy number. qRT-PCR amplification was performed using an ABI 7300 Real-Time PCR Detection System with SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). Cycling conditions were 95°C for 10 minutes followed by 40 repeats of 95°C for 15 seconds and 60°C for 1 minute. Relative amounts of mtDNA copy number were normalized to the nuclear 18S rRNA gene, and mRNA was normalized to GAPDH, and were calculated using delta method from threshold cycle numbers.

Immunofluorescence staining for PEA3

For immunofluorescence analysis of PEA3 expression, kidneys from all groups were harvested and embedded in Tissue-Tek OCT medium (Sakura Finetek Europe, Zoeterwoude, The Netherlands) and snap-frozen in liquid nitrogen-cooled isopentane. Sections (5 µm) were obtained in a cryostat, collected on slides, and fixed for 10 min in 1:1 methanol/acetone solution at room temperature, then store at -20°C. Sections were rehydrated in PBS at room temperature for 10 min. Incubation of the tissue sections was performed overnight at 4°C with the rabbit anti-PEA3 polyclonal antibody (1:50 in PBS). Negative controls were prepared for each sample by following the same staining procedure in the absence of primary antibodies. After one short and three 10-minute washes in PBS, the slides were incubated for 1 hour at 37°C with a goat FITC-conjugated anti-rabbit antibody (1:30 in PBS). Then after three 10-minute washes with PBS, slides were mounted using 40% glycerol. Immunofluorescence was visualized with an Olympus microscope by the emission of FITC fluorescence (200 x magnification), and images were processed using JEDA 801D Vision 6.0 software (China).

Cell culture

MPTCs (mouse proximal tubular cells) were grown and propagated at 37°C in MEM/F12 (Wisent Inc. Montreal, CA) with 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA).

Transient transfection of PEA3 plasmid and PEA3 siRNA

MPTCs were cultivated to 60% to 70% confluence in culture medium containing no penicillin or streptomycin. pcDNA3.1-PEA3 plasmid and empty vector (Genechem Inc. Shanghai, China), or PEA3-siRNA and control siRNA (Genechem Inc. Shanghai, China) were transfected into the cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol. After 20 h, gentamicin (1 mg/ml) were added to the cells for another 24 h.

Apoptosis analysis

Annexin V-fluorescein isothiocyanate conjugated with propidium iodide staining was used for apoptosis analysis. After the treatments, MPTCs were seeded into six-well plates and the apoptosis was quantified by flow cytometry using annexin V-fluorescein isothiocyanate and propidium iodide double staining (annexin V: fluorescein isothiocyanate apoptosis detection kit, BD Biosciences, San Diego, CA), according to the manufacturer's instructions.

Measurement of mitochondrial membrane potential

The MMP of MPTCs were monitored using JC-1, a MMP-sensitive fluorescent dye, as described previously [15]. Briefly, the isolated mitochondrial pellet and dissociated MPTCs were washed twice with Hank's balanced salt solution (Sigma), then incubated in the dark with JC-1 (7.5 mmol/l; 30 min at 37°C). Cells were washed with JC-1 washing buffer, and detected by fluorescence-assisted cell sorting for MPTCs. The relative MMP was calculated using the ratio of J-aggregate/monomer (590/520 nm). Values are expressed as the fold-increase in J-aggregate/monomer fluorescence over the control cells.

Detection of mitochondrial ROS

Mitochondrial ROS production in the MPTC was assessed by MitoSOX Red mitochondrial super-

PEA3 and gentamicin nephrotoxicity

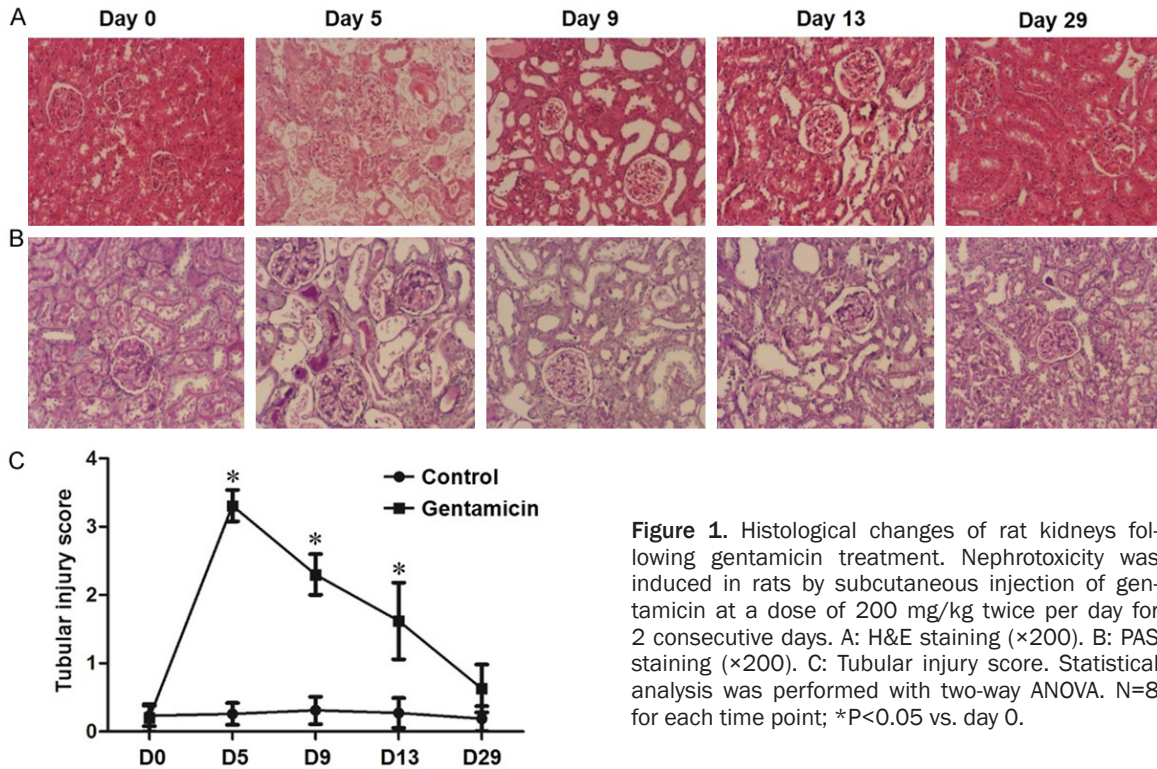


Figure 1. Histological changes of rat kidneys following gentamicin treatment. Nephrotoxicity was induced in rats by subcutaneous injection of gentamicin at a dose of 200 mg/kg twice per day for 2 consecutive days. A: H&E staining ($\times 200$). B: PAS staining ($\times 200$). C: Tubular injury score. Statistical analysis was performed with two-way ANOVA. N=8 for each time point; * $P < 0.05$ vs. day 0.

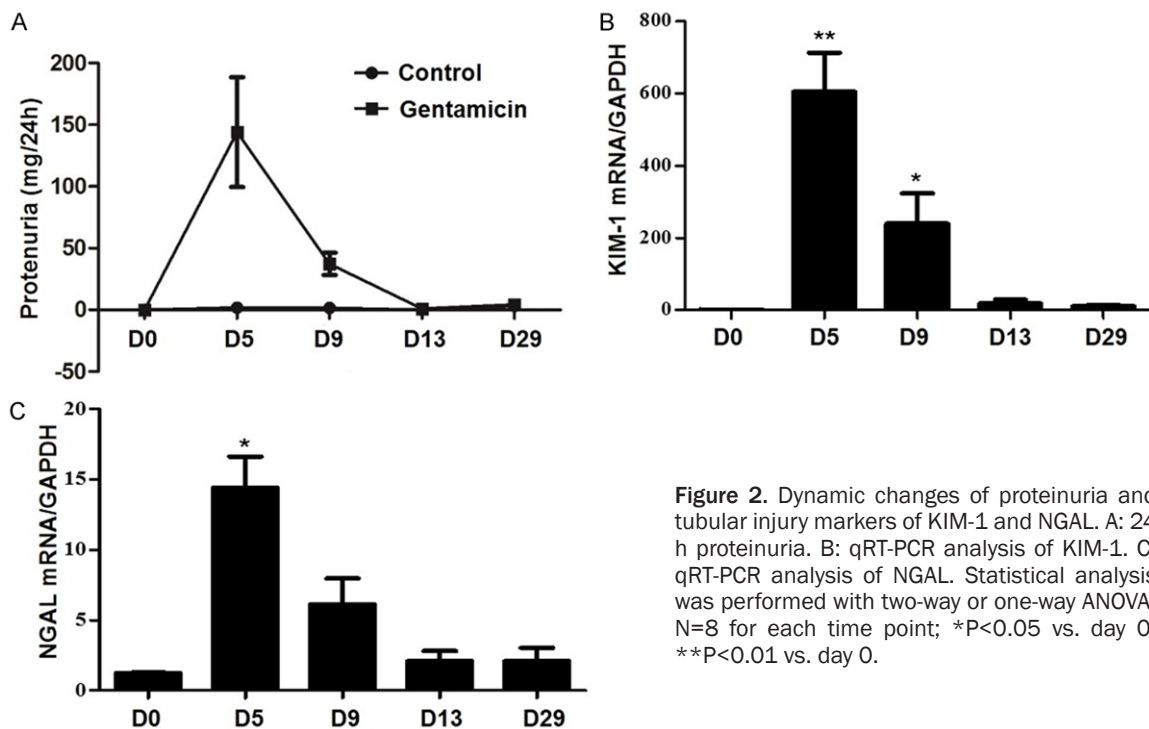


Figure 2. Dynamic changes of proteinuria and tubular injury markers of KIM-1 and NGAL. A: 24 h proteinuria. B: qRT-PCR analysis of KIM-1. C: qRT-PCR analysis of NGAL. Statistical analysis was performed with two-way or one-way ANOVA. N=8 for each time point; * $P < 0.05$ vs. day 0, ** $P < 0.01$ vs. day 0.

oxide indicator (Invitrogen, Carlsbad, CA, USA). The MPTCs were seeded into the 6-well plate and grown to a density of 1×10^6 cell/ml. After

incubation with HSYA and/or AKBA for 24 h, the cells were added with 5 μ M MitoSOX Red for 15 min at 37°C in a CO₂ incubator. Then the cells

PEA3 and gentamicin nephrotoxicity

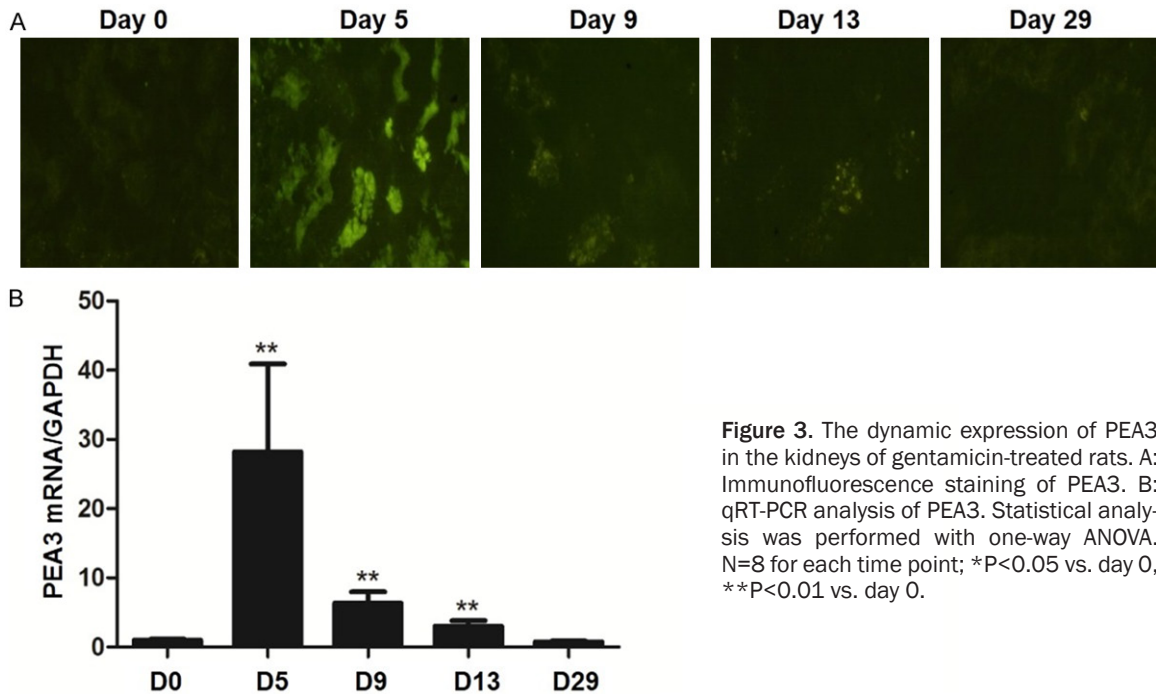


Figure 3. The dynamic expression of PEA3 in the kidneys of gentamicin-treated rats. A: Immunofluorescence staining of PEA3. B: qRT-PCR analysis of PEA3. Statistical analysis was performed with one-way ANOVA. N=8 for each time point; *P<0.05 vs. day 0, **P<0.01 vs. day 0.

were carefully washed twice with PBS. Fluorescence was read at 510 nm (excitation) and 580 nm (emission). The fluorescence intensity was quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

Data were presented as means \pm SE. Statistical analysis was performed using ANOVA followed by a Bonferroni posttest or unpaired Student's t-test using SPSS 13.0 statistical software. P<0.05 were considered statistically significant difference.

Results

Dynamic expression of renal PEA3 in rats with gentamicin-induced nephrotoxicity

Firstly, we established a rat model of gentamicin nephrotoxicity. As shown by **Figure 1A-C**, gentamicin induced a severe renal tubular injury on day 5. Then the renal injury gradually recovered after gentamicin administration for 29 days. The necrosis mainly occurred in the proximal tubular epithelium with intense granular degeneration, tubular lumen dilation, and cast formation (**Figure 1A** and **1B**). Compared to the control animals (day 0), gentamicin also caused severe proteinuria on day 5 (144.00 ± 44.44 mg/24 h), which was attenuated on day

9 (37.29 ± 9.13 mg/24 h) and entirely recovered after 13 days (**Figure 2A**). In parallel with the severity of renal morphological damage and proteinuria, the renal tubular injury markers of kidney injury molecule 1 (KIM-1) and neutrophil gelatinase-associated lipocalin (NGAL) [16] were accordingly elevated as determined by qRT-PCR (**Figure 2B** and **2C**).

Next, we examined the dynamic expression of PEA3 in the kidneys of rats with or without gentamicin treatment. By immunofluorescence staining, we could not find a positive staining in the kidneys of normal rats (**Figure 3A**), while gentamicin induced a striking upregulation of PEA3 in renal tubules on day 5. After 5 days, PEA3 expression gradually decreased and finally returned to the normal level on day 29 (**Figure 3A**). Consistently, qRT-PCR detected a similar pattern of PEA3 mRNA expression as its protein regulation (**Figure 3B**). Interestingly, the pattern of PEA3 regulation during this experimental process closely followed the dynamic changes of renal tubular injury, suggesting a possible role of PEA3 in gentamicin nephrotoxicity.

Overexpression of PEA3 in MPTCs mitigated gentamicin-induced MPTC injury and MtD

To define the role of PEA3 in the pathogenesis of gentamicin nephrotoxicity, we further estab-

PEA3 and gentamicin nephrotoxicity

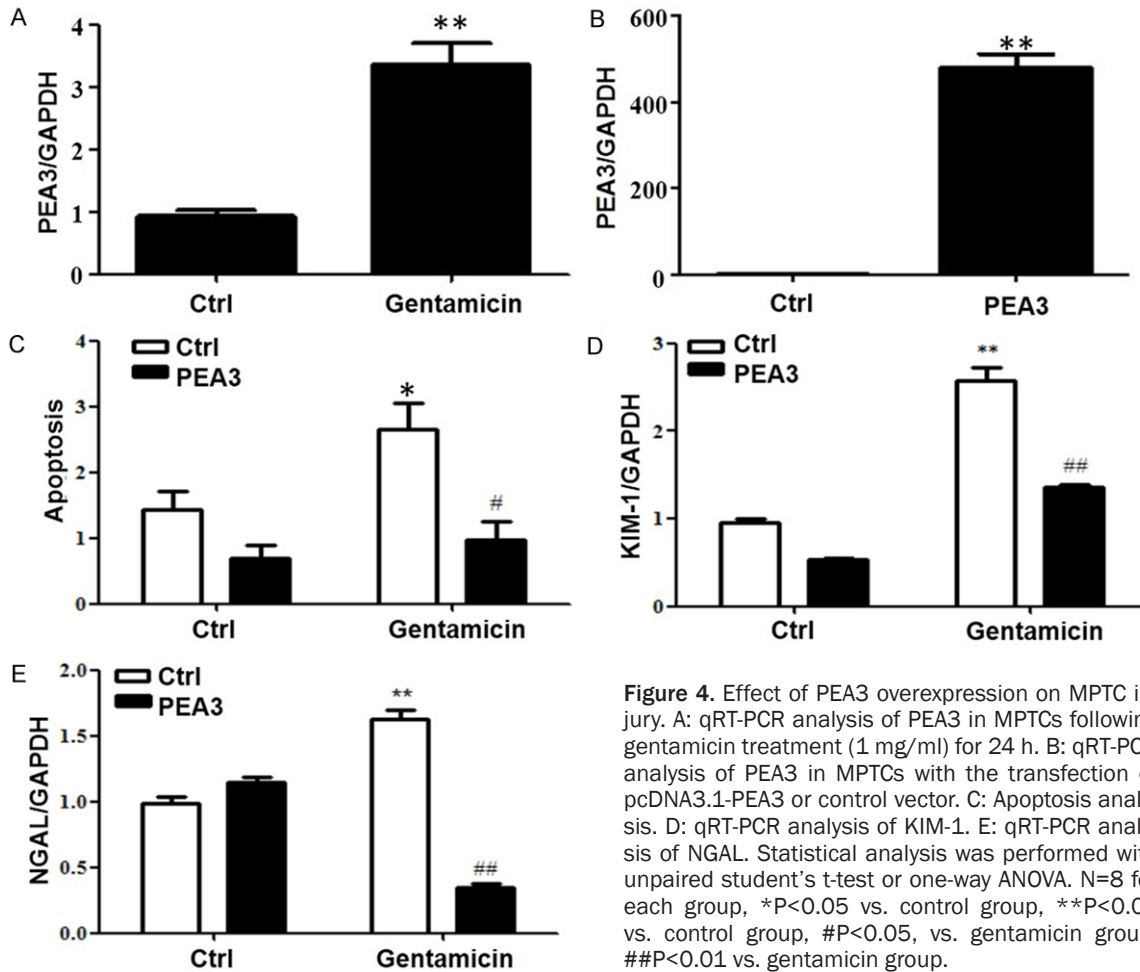


Figure 4. Effect of PEA3 overexpression on MPTC injury. A: qRT-PCR analysis of PEA3 in MPTCs following gentamicin treatment (1 mg/ml) for 24 h. B: qRT-PCR analysis of PEA3 in MPTCs with the transfection of pcDNA3.1-PEA3 or control vector. C: Apoptosis analysis. D: qRT-PCR analysis of KIM-1. E: qRT-PCR analysis of NGAL. Statistical analysis was performed with unpaired student's t-test or one-way ANOVA. N=8 for each group, *P<0.05 vs. control group, **P<0.01 vs. control group, #P<0.05, vs. gentamicin group, ##P<0.01 vs. gentamicin group.

lished a gentamicin-induced renal tubular cell injury model. As shown by data, in MPTCs, gentamicin enhanced PEA3 mRNA expression by 3.6-fold (Figure 4A). Meantime, it caused obvious cell apoptosis in parallel with the upregulation of injury markers of KIM-1 and NGAL (Figure 4C-E). Then we overexpressed PEA3 in MPTCs by transfecting pcDNA3.1-PEA3 plasmid (Figure 4B) and found that the gentamicin-induced cell apoptosis and the upregulation of KIM-1 and NGAL were largely normalized by overexpressing PEA3 (Figure 4C-E).

MtD has been shown as a pathogenic factor in renal diseases [17, 18]. Thus, we examined the mitochondrial function in MPTCs following gentamicin treatment with or without PEA3 overexpression. As expected, gentamicin induced significant MtD as evidenced by the decreased mitochondrial membrane potential (MMP), reduced mtDNA copy number, and increased reactive oxygen species (ROS) production (Figure 5A-D). Strikingly, PEA3 overexpression remarkably ameliorated gentamicin-induced

MtD (Figure 5A-D). These data demonstrated that overexpressing PEA3 prevented gentamicin-induced MPTC injury in line with a protection of mitochondrial function.

Silencing PEA3 deteriorated gentamicin-induced MPTC injury and MtD

Finally, PEA3 siRNA was applied to the MPTCs for the further confirmation of PEA3 effects on gentamicin-induced renal tubular cell injury and MtD. As shown by the data, PEA3 siRNA application further promoted gentamicin-induced cell apoptosis (Figure 6A and 6B). In addition, the MtD induced by gentamicin was further deteriorated by PEA3 silencing (Figure 7A-D). These data further confirmed the protective role of PEA3 against gentamicin-induced renal tubular cell injury and MtD.

Discussion

As reported, many genes acting on nephrogenesis also contribute to the repair process after

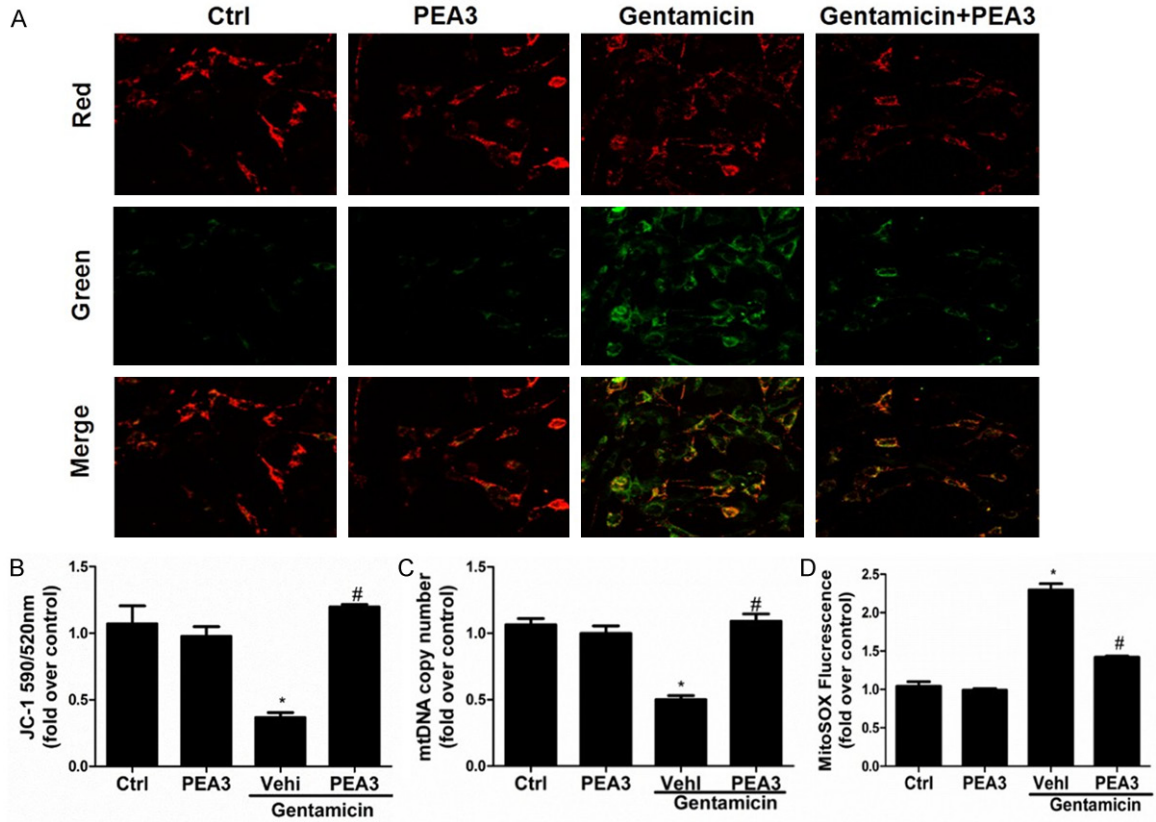


Figure 5. Effect of PEA3 overexpression on mitochondrial function in gentamicin-treated MPTCs. MPTCs were transfected with pcDNA3.1-PEA3 or control vector for 20 h. Then gentamicin (1 mg/ml) were added to the cells for another 24 h. A: Representative images of MPTCs stained with JC-1. B: Mitochondrial membrane potential (MMP), C: Mitochondrial DNA (mtDNA) copy number, D: MitoSOX fluorescence. Statistical analysis was performed with one-way ANOVA. N=6 for each group. *P<0.05 vs. PEA3 and control group. #P<0.05 vs. gentamicin group.

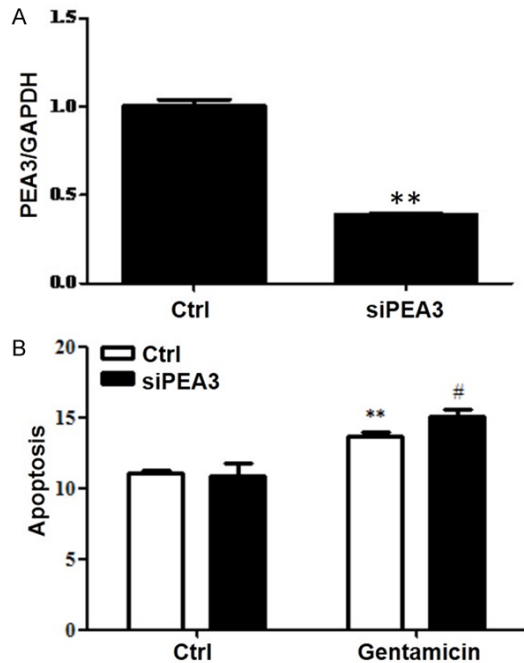


Figure 6. Effect of PEA3 silencing on gentamicin-induced MPTC apoptosis. MPTCs were transfected with siPEA3 or control siRNA for 20 h. Then gentamicin (1 mg/ml) were added to the cells for another 24 h. A: qRT-PCR analysis of PEA3. B: Apoptosis analysis. Statistical analysis was performed with unpaired student's t-test or one-way ANOVA. N=6 for each group. **P<0.01 vs. control. #P<0.05 vs. gentamicin group.

AKI [19-21]. Evidence demonstrated that PEA3 played an important role in the nephrogenesis. However, the role of PEA3 in the tubular injury of AKI is still unknown. In the recent decades, gentamicin-induced AKI model has been widely used to investigate the mechanisms and therapies of AKI [22, 23]. The typical pathological change of gentamicin-induced AKI was the tubular injury but not the glomerular damage. In the present study, gentamicin was administered to the rats and MPTCs to induce renal tubular injury. In animals, gentamicin caused massive tubular necrosis, hyaline casts, signifi-

PEA3 and gentamicin nephrotoxicity

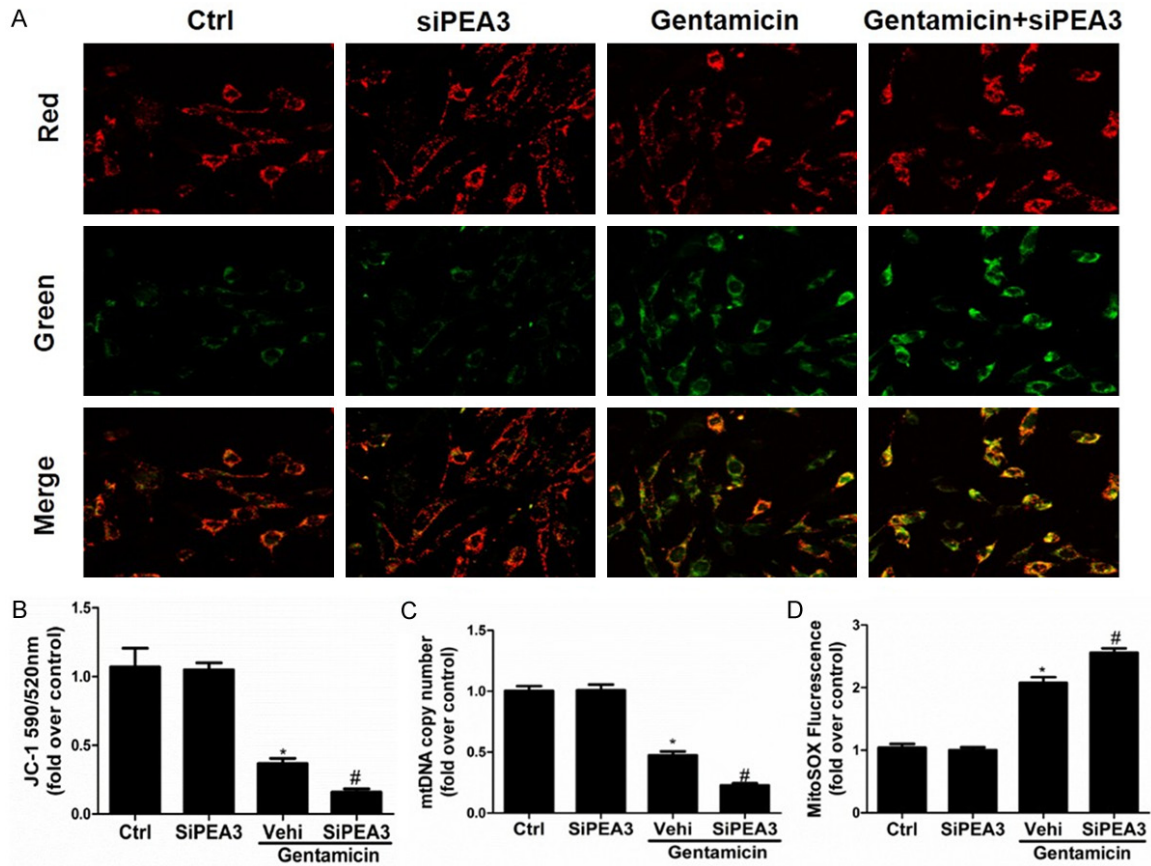


Figure 7. Effects of PEA3 silencing on gentamicin-induced MtD in MPTCs. MPTCs were transfected with PEA3 siRNA or control siRNA for 20 h. Then gentamicin (1 mg/ml) were added to the cells for another 24 h. A: Representative images of MPTCs stained with JC-1. B: Mitochondrial membrane potential (MMP), C: Mitochondrial DNA (mtDNA) copy number, D: MitoSOX fluorescence. Statistical analysis was performed with one-way ANOVA. N=6 for each group. *P<0.05 vs. PEA3 or control group. #P<0.05 vs. gentamicin group.

cant proteinuria, and enhanced expressions of KIM-1 and NGAL, characterizing a model of nephrotoxic AKI. Meantime, the induction of PEA3 in the renal tubules of gentamicin-treated rats showed a similar dynamic pattern as tubular injury. These results suggested a potential that PEA3 might be of importance in gentamicin nephrotoxicity.

To explore the contribution of PEA3 in gentamicin nephrotoxicity, we overexpressed PEA3 in MPTCs with or without gentamicin treatment. Compared to the controls, overexpression of PEA3 alleviated the upregulation of tubular injury markers of KIM-1 and NGAL in line with the attenuation of cell apoptosis. In agreement with these results, inhibition of PEA3 by a siRNA strategy led to more severe injury in gentamicin-treated MPTCs. These results indicated a protective role of PEA3 against gentamicin-induced renal tubular cell injury.

Mitochondrial dysfunction (MtD) was characterized by the dysfunction of membrane-bound oxidative phosphorylation (OxPhos) complexes (Cxs) in mitochondria, leading to the reduction of ATP, accumulation of reactive oxygen species (ROS), and depletion of mitochondrial DNA [24]. The alteration of mtDNA copy number, mitochondrial ROS, MMP were usually used as the markers of mitochondrial dysfunction [25, 26]. By reviewing the literatures, studies suggested that mitochondria dysfunction played a central role in tissue damage caused by gentamicin [27, 28]. It is also known that mitochondria are the main intracellular sites for ROS production. Mitochondrial damage could lead to more ROS production, promoting the expression of pro-inflammatory cytokines and apoptotic response, which ultimately results in tubular injury [28, 29]. In agreement with this notion, Andoh et al. reported that PEA3-binding motifs con-

tributed to the protection against oxidative injury and apoptosis through *NO/PKG/thioredoxin (Trx)* pathway [30]. Here we examined mitochondrial function and found that gentamicin caused remarkable mitochondrial abnormality in MPTCs, which was markedly attenuated by PEA3 overexpression. Using a PEA3 siRNA approach, gentamicin-induced MtD was further deteriorated in renal tubular cells. These results highly suggested that the PEA3 action in protecting mitochondria could be a potential mechanism mediating the protective role of PEA3 against gentamicin nephrotoxicity to some extent.

In summary, in the present study, we reported a dynamic alteration of renal PEA3 in animals with gentamicin nephrotoxicity for the first time. Also, employing the genetic approaches, we firstly provided the evidence showing a protective role of PEA3 in the pathogenesis of gentamicin-induced renal tubular cell injury and MtD. These findings not only suggested a novel molecular mechanism mediating the gentamicin nephrotoxicity but also introduced a new candidate into the research field of AKI.

Acknowledgements

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Disclosure of conflict of interest

None.

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PEA3 and gentamicin nephrotoxicity

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