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NEPHROLOGY - ORIGINAL PAPER

Intervention of mitochondrial activity attenuates cisplatin-induced acute kidney injury

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Abstract

Objectives The dysfunction of mitochondrial respiratory chain induced by cisplatin results in overproduction of reactive oxygen species (ROS) which contributes to kidney injury. The current study aimed to evaluate the effect of a mitochondrial electron transport inhibitors of rotenone (mitochondrial complex I inhibitor) and azoxystrobin (mitochondrial complex III inhibitor), in cisplatin-induced kidney injury.

Methods In vivo, cisplatin was administered to male C57BL/6J mice by a single intraperitoneal (i.p.) injection (20 mg/kg). Then the mice were treated with or without 200 ppm rotenone in food. Mice were sacrificed after cisplatin administration for 72 h. The serum and the kidney tissues were collected for further analysis. In vitro, mouse proximal tubular cells (mPTCs) were treated with cisplatin (5 μg/mL) and rotenone/azoxystrobin for 24 h. Flow cytometry, Western blotting, and TUNEL staining were used to evaluate the cell injury.

Results In vivo, rotenone treatment obviously ameliorated cisplatin-induced renal tubular injury evidenced by the improved histology and blocked NGAL upregulation. Meanwhile, cisplatin-induced renal dysfunction shown by the increased levels of serum creatinine (Scr), blood urea nitrogen (BUN), and cystatin C were significantly reduced by rotenone treatment. Moreover, the increments of cleaved caspase-3 and transferase dUTP nick-end labeling (TUNEL)-positive cells were markedly decreased in line with the attenuated mitochondrial dysfunction and oxidative stress after rotenone administration. In vitro, rotenone and azoxystrobin protected against mitochondrial dysfunction, oxidative stress, and renal tubular cell apoptosis induced by cisplatin.

Conclusions Our results demonstrated that inhibition of mitochondrial activity significantly attenuated cisplatin nephrotoxicity possibly by inhibiting mitochondrial oxidative stress.

Keywords Cisplatin · AKI · Mitochondrial activity · Oxidative stress

Introduction

Cis-Diamminedichloroplatinum (cisplatin), which was discovered accidently five decades ago, is one of the most widely used anticancer agents for the treatment of various types of solid tumors [1]. However, its serious adverse effects in normal tissues, particularly irreversible nephrotoxicity limit the clinical application of this drug [2, 3]. Although the underlying mechanisms of cisplatin-induced kidney injury have been documented in the past decades [2, 4], the details are still not fully understood.

Recently, accumulating evidence indicated that mitochondrial dysfunction was associated with many types of kidney diseases including cisplatin-induced kidney injury [5, 6]. Renal tubule cells are rich in mitochondria [7]. Mitochondria

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are the key source of energy production and play important roles in cell signaling transduction, cell proliferation, cell growth, and cell death [8, 9]. Several studies suggested that mitochondria are an important target organelle in cisplatin-induced kidney injury [10, 11]. The mitochondria dysfunction induced by cisplatin can be displayed by the reduced mitochondrial membrane potential, ATP depletion, reactive oxygen species (ROS) overproduction, and release of proapoptotic factors such as mitochondrial DNA and cytochrome C, leading to renal tubule injury and cell death [12]. Therefore, targeting the mitochondrial dysfunction could be beneficial for protecting against cisplatin nephrotoxicity.

In the past decades, several possible mechanisms have been proposed to account for ROS overproduction induced by cisplatin [2]. First, cisplatin is converted into a highly reactive form after transported into cell, which can rapidly react with thiol-containing molecules such as glutathione, resulting in the accumulation of endogenous ROS and oxidative stress [13]. Second, cisplatin disrupts the mitochondrial respiratory chain to enhance ROS production [14]. However, the therapeutic strategy based on ROS regulation is still unsatisfactory in clinical trials of AKI therapy possibly owing to the incomplete understanding on the mechanisms of ROS generation and ROS action in AKI [15]. Our previous study showed that treatment with rotenone, a mitochondrial complex-1 inhibitor, could improve the mitochondrial dysfunction and attenuate the kidney injury in UUO and I/R animal models [16, 17]. These results suggested overactive mitochondrial activity may play an important role in mitochondrial dysfunction and ROS overproduction in the pathogenesis of chronic kidney disease [17]. However, the relationship between mitochondrial activity and the axis of mitochondrial oxidative stress/kidney injury in cisplatin nephrotoxicity needs to be investigated.

Materials and methods

Animal model of cisplatin-induced AKI

Wild-type C57BL/6J mice were obtained from animal core facility of Nanjing Medical University (Nanjing, China) and were maintained on a 12-h light and 12-h dark cycle in a standard SPF animal room. To evaluate the effect of rotenone on cisplatin-induced acute injury, 8-week-old male C57BL/6J mice were divided into 3 groups: control group (control; $n=8$), cisplatin treatment group (cisplatin; $n=8$), and cisplatin plus rotenone group (cisplatin + rotenone; $n=8$). In this study, the mice were not fed but allowed free access to water for 18 h prior to the experiments. Cisplatin (Sigma-Aldrich, 15663-27-1) was freshly prepared in saline at a concentration of 2 mg/mL, then administered to the mice by a single intraperitoneal (i.p.) injection (20 mg/kg), in both cisplatin and cisplatin + rotenone groups. The control mice received an i.p. injection of equal volume of saline. After cisplatin injection, the mice were fed with jelly diet with or without rotenone (Sigma-Aldrich, R8875) at a dose of 200 ppm. After 72-h cisplatin administration, mice were sacrificed, and serum was obtained from blood samples, then stored at − 80 °C for further analysis. For histology analysis, the kidney tissues were collected and fixed in 4% paraformaldehyde (PFA) immediately. All of the remaining kidney tissue were collected and stored at − 80 °C for mRNA and protein analysis. The levels of serum creatinine and blood urea nitrogen were measured by using an automatic biochemical analyzer in Children's Hospital of Nanjing Medical University. All animal experiments were performed according to the protocols approved by the Institutional Animal Care and Use Committee of Nanjing Medical University (IACUC 14030112-2).

Cell culture and treatment

Mouse proximal tubular cells (mPTCs) obtained from American Type Culture Collection (ATCC, Manassas, VA) were cultured in DMEM/F-12 medium (Gibco, china, 319-075-CL) supplemented with 10% fetal bovine serum (GIBCO, 26170035), 100 U/mL penicillin, and 100 μ g/mL streptomycin at 37 °C in a humidified atmosphere of 5% CO2 (Invitrogen, Carlsbad CA, USA). When the cells were grown to 70% confluence, rotenone (10, 20, 40 nM) or azoxystrobin (Sigma-Aldrich, 31697, 1 μM) together with cisplatin (5 μg/mL) were added to the serum-free medium to treat mPTCs for 24 h.

Quantitative real-time PCR (qRT-PCR)

TRIzol (TAKARA, Dalian, China; 9108) was used to extract total RNA from kidney tissues and cells. Then, the first-strand cDNAs were synthesized from 1 μg of total RNAs in a 20 μL reaction using a Reverse Transcription M-MLV kit (TAKARA, 2641A) following the manufacturer's instructions. Quantitative real-time PCR was performed in Applied Biosystems 7500 Real Time PCR System using SYBR Green PCR mix (Vazyme, Nanjing, China; q111-02/03). Primers were synthesized by Generay Biotech (Shanghai, China) and the sequences were listed in Table 1. The PCR cycling condition was 95 °C for 10 min, followed by 40 repeats of 95 °C for 15 s and 60 °C for 1 min. β-actin was chosen as an internal control. The relative levels of mRNA expression were analyzed and calculated relative to threshold cycle values (ΔCt), then converted to fold changes using $2^{-\Delta\Delta Ct}$ method as described previously [18].

Table 1 Primer sequences

Gene	Primer sequence (5′–3′)
Mouse MCP-1	F: GCTCTCTCTTCCTCCACCAC R: ACAGCTTCTTTGGGACACCT
Mouse IL-1β	F: ACTGTGAAATGCCACCTTTTG R: TGTTGATGTGCTGCTGTGAG
Mouse IL-6	F: ACAAAGCCAGAGTCCTTCAGAGAG R: TTGGATGGTCTTGGTCCTTAGCCA
Mouse TNF-α	F: TCCCCAAAGGGATGAGAAG R: CACTTGGTGGTTTGCTACGA
Mouse Cox-2	F: AGGACTCTGCTCACGAAGGA R: TGACATGGATTGGAACAGCA
Mouse VCAM-1	F: GGAGAGACAAAGCAGAAGTGG R: AACACAAGCGTGGATTTGG
Mouse mt-ND1	F: ACACTTATTACAACCCAAGAACACAT R: TCATATTATGGCTATGGGTCAGG
Mouse mt-ND3	F: CCCCAAATAAATCTGTA R: CTCATGGTAGTGGAAGT
Mouse mt-ND5	F: GCCAACAACATATTTCAACTTTTC R: ACCATCATCCAATTAGTAGAAAGGA
Mouse mt-COX1	F: CAGACCGCAACCTAAACACA R: TTCTGGGTGCCCAAAGAAT
Mouse mt-COX2	F: GCCGACTAAATCAAGCAACA R: CAATGGGCATAAAGCTATGG
Mouse mt-ATP6	F: CCATAAATCTAAGTATAGCCATTCCAC R: AGCTTTTTAGTTTGTGTCGGAAG
Mouse mt-ATP8	F: ACATTCCCACTGGCACC R: GGGGTAATGAATGAGGC
Mouse TFAM	F: AGCTGTGAGCAAGTATAAAGAGCA R: TCAGGAGACAGATTTTTCCAAG
Mouse β-actin	F: GAGACCTTCAACACCCCAGC R: ATGTCACGCACGATTTCCC

Western blotting analysis

The kidney tissues or cells were lysed in protein lysis buffer (50 mM Tris–HCl, 250 mM NaCl, 0.5% Triton X-100, 50 mM NaF, 2 mM EDTA and 1 mM Na3VO4) supplemented with 1 × EDTA free protease inhibitor cocktail (Roche, 04693132001) for 20 min on ice. Protein concentration was measured using the Bradford method, then 30 μg total protein was used for immunoblotting analysis following standard methods with primary antibodies against Bax (Cell Signaling Technology; 2772, 1:1000), cleaved caspase 3 (Cell Signaling Technology; 9661, 1:1000), NGAL (Abcam; ab63929, 1:1000), ATPB (Proteintech; 17247-1-AP, 1:1000), SOD2 (Proteintech; 24127-1-AP, 1:1000), β-actin (Biogot; AP0060, 1:1000), followed by the incubation with peroxidase-conjugated goat anti-rabbit secondary antibody (Beyotime; A0208, 1:1000). The immunoblotted bands were detected using the enhanced chemiluminescence detection system (Bio-Rad, Hercules, CA, USA). Densitometric analysis was performed by using Image J (Wayne Rasband National Institutes of Health, USA).

Cell apoptosis assay

After treatment, mPTCs were washed for three times with cold PBS, then trypsinized and adjusted to 5×10^4/mL and double-stained with annexin V-FITC and PI using an apoptosis detection Kit (BD Biosciences, 556547, San Diego, CA) according to the manufacturer's instructions. After incubation for 20 min at room temperature in the dark, the fluorescent intensity was measured using a flow cytometer (BD Biosciences, San Diego, CA).

Enzyme linked immunosorbent assay (ELISA)

The protein levels of circulating inflammatory factors including TNF-α (DKW12-2720-096), IL-1β (DKW12-2012-096), IL-6 (DKW12-2060-096), and MCP-1 (DKW12-2739-096) were detected by the ELISA kits (DAKEWEI, Shenzhen, China) according to the manufacturer's instructions. The levels of serum Cystatin C were also determined by a mouse Cystatin C ELISA kit (E-EL-M0389C, Elascience, China).

Immunohistochemistry

Firstly, the kidney sections (4 μm thick) were mounted on slides. Then the slides were boiled in 500 ml 1 × improved Citrate Antigen Retrieval Solution (Beyotime, P0083) for 1 min and cooled on bench top for 20 min. After incubation with 3% hydrogen peroxide for 15 min, sections were blocked with 10% normal goat serum for 60 min at 37 °C and then incubated with primary mouse monoclonal antibody against TNF-α (GB11188, 1:100; Servicebio), MCP-1 (GB11199, 1:100; Servicebio), and NGAL (Abcam; ab63929, 1:100) for overnight at 4 °C. After washing with PBST buffer for three times, sections were incubated with horseradish peroxidase-conjugated secondary antibody for 60 min. Localization of peroxidase conjugates was determined using a DAB kit (ZLI-9018, zsbio, China).

Renal histology and tubular injury scoring

Periodic acid-Schiff (PAS) staining was used to analyze renal histology. The tubular damage was indicated by tubular lysis, dilation, disruption, necrosis, and cast formation (× 400 magnification). Tubular injury scoring was graded by a semiquantitative score from 0 to 4 +: 0, no abnormalities; 1 +, changes affecting less than 25% of the sample; 2 +, changes affecting 25–50%; 3 +, changes affecting 50–75%; 4 +, changes affecting more than 75% [19].

Mitochondrial membrane potential

The MMP of mPTCs was monitored using a mitochondrial membrane potential assay kit with JC-1 according to the

manufacturer's instructions. Briefly, the mPTCs were incubated with JC-1 working solution in the dark for 30 min at 37 °C. Then the cells were washed with JC-1 washing buffer, and fluorescence was detected by flow Cytometry. The relative MMP was calculated using the ratio of J-aggregate/monomer (590/520 nm) as described previously [20].

Measurement of malondialdehyde (MDA)

Malondialdehyde (MDA) is a natural product of lipid oxidation, and the level of MDA indicates the level of lipid oxidation. In this study, the measurement of MDA was based on the reaction with thiobarbituric acid by using a commercially available lipid peroxidation MDA Assay Kit (Beyotime; S0131) according to the manufacturer's instructions.

Measurement of ROS production

The level of ROS production in mPTCs induced by cisplatin was measured using a commercially available Reactive Oxygen Species Assay Kit (Beyotime; S0033) according to the manufacturer's instructions.

TUNEL assays

In situ cell death was detected using a TUNEL BrightGreen Apoptosis Detection Kit as instructed by the manufacturer (A112-01/02/03, Vazyme, China). After TUNEL staining, the slides were observed by confocal microscopy. Five randomly visual fields of blinded samples were selected and the number of apoptotic cells was counted.

Statistical analysis

The data were presented as the mean value ± S.D. Statistical significance was determined by ANOVA analysis or the unpaired Student's *t*-test using GraphPad Prism (version 6.0, GraphPad Software, La Jolla, CA, USA) software. A value of $P < 0.05$ was considered statistically significant.

Results

Rotenone treatment alleviated acute kidney injury induced by cisplatin

The renal function following 3 days of cisplatin treatment was significantly impaired as shown by increased serum creatinine (from 5.28 ± 1.64 to 168.64 ± 35.27 μM, $P < 0.01$) (Fig. 1a), blood urea nitrogen (from 9.71 ± 0.71 to 84.56 ± 15.11 mM, $P < 0.01$) (Fig. 1b), and serum cystatin C (from 7.55 ± 1.81 to 19.91 ± 1.26 ng/ml, $P < 0.01$) (Fig. 1c). PAS staining indicated marked tubular structure damage shown by tubular cell necrosis, renal tubule dilation, and protein cast formation (Fig. 1d). Strikingly, 3-day rotenone treatment remarkably improved renal function as shown by reduced serum creatinine (from 168.64 ± 35.27 to 88.64 ± 55.91 mM, $P < 0.05$)

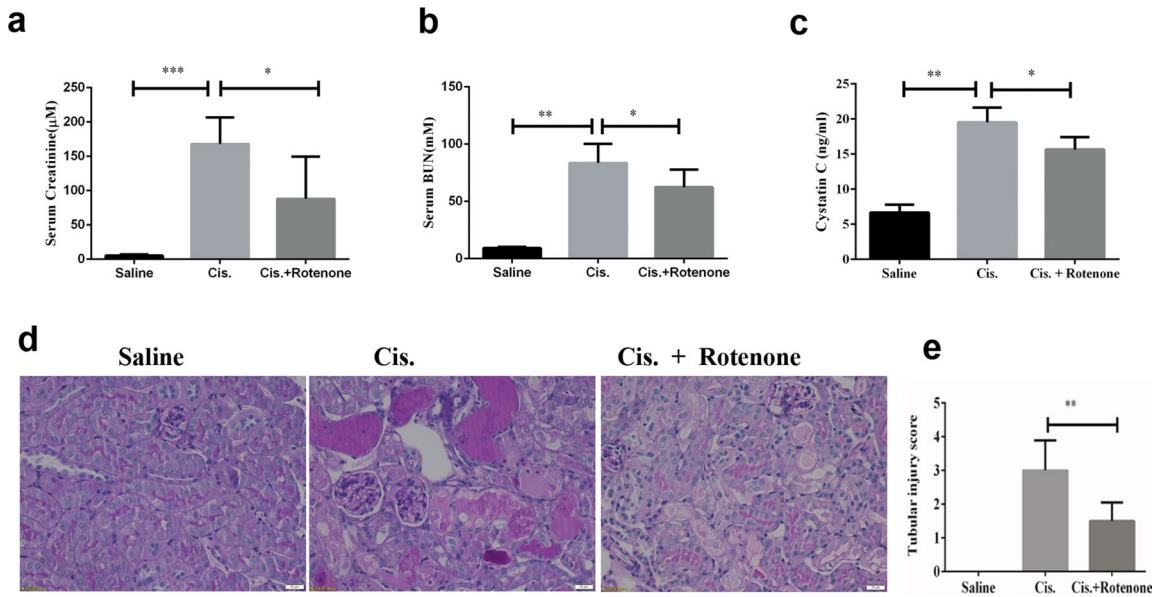

Fig. 1 Rotenone treatment alleviated acute kidney injury induced by cisplatin. Serum creatinine (**a**), blood urea nitrogen (**b**), and serum cystatin C levels (**c**). **d** Representative images of periodic acid-Schiff staining (magnification × 400) of kidneys. **e** Tubular injury score analysis. Data were presented as means ± S.D. of 10 random fields from each slide ($n = 8$ in each group). Cis: cisplatin; ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$

(Fig. 1a), blood urea nitrogen (from 84.56 ± 15.11 to 62.41 ± 14.21 mM, $P < 0.05$) (Fig. 1b), and serum cystatin C (from 19.91 ± 1.26 to 15.61 ± 1.26, $P < 0.05$) (Fig. 1c). Meanwhile, rotenone treatment also significantly attenuated morphological abnormalities (Fig. 1d, e).

To further clarify the protective role of rotenone in cisplatin-induced renal tubule injury, we examined the protein levels of neutrophil gelatinase-associated lipocalin (NGAL) which is a more sensitive tubular injury marker. Immunohistochemistry and Western blotting data showed that the enhanced protein levels of NGAL were remarkably reduced by rotenone treatment (Fig. 2a–c). As for the rotenone toxicity in mice, our previous studies showed that treatment with rotenone alone at a dose of 200–500 ppm in food did not cause side effects in major organs including kidney, liver, and heart [16, 17]. All of these data suggested that rotenone treatment could attenuate renal dysfunction and pathological damage induced by cisplatin.

Rotenone treatment blunted apoptosis induced by cisplatin

In this study, the protein levels of Bax and cleaved caspase-3 were analyzed by western blotting. The results showed that the enhanced levels of Bax and cleaved caspase-3 induced by cisplatin were markedly suppressed by rotenone treatment (Fig. 3a, b). Moreover, the increased number of TUNEL-positive cells in renal tubules of cisplatin-treated mice was obviously lowered after rotenone treatment (Fig. 3c, d). These data suggested that rotenone treatment could prevent tubular cell apoptosis in cisplatin-induced nephrotoxicity.

Rotenone treatment ameliorated inflammatory response induced by cisplatin

In this study, the effect of rotenone treatment on cisplatin-induced renal inflammation was also analyzed. As shown in Fig. 4a, the mRNA expressions of inflammatory factors including MCP-1, TNF-α, IL-6, IL-1β, VCAM-1, and COX-2 were all markedly increased in the kidneys of mice challenged with cisplatin, which was significantly blunted after rotenone treatment. Additionally, ELISA data showed that the enhanced protein levels of TNF-α, IL-1β, IL-6, and MCP-1 in the circulation of cisplatin-treated mice were significantly reduced by rotenone (Fig. 4b–e). Furthermore, by immunohistochemistry, we further confirmed that rotenone treatment downregulated the protein levels of MCP-1 and TNF-α in renal tubules induced by cisplatin (Fig. 4f, g). These data indicated that rotenone treatment ameliorated inflammatory response caused by cisplatin.

Rotenone treatment attenuated mitochondrial dysfunction and oxidative stress in mice induced by cisplatin

Given that rotenone is a mitochondrial complex I inhibitor by inhibiting mitochondrial electron transport, we examined the role of rotenone in cisplatin-induced mitochondrial dysfunction. The mRNA levels of multiple mitochondrial-encoded genes were detected by real-time PCR. As shown by the data (Fig. 5a), the mRNA levels of mitochondrial DNA-encoded genes including *mt-Nd1,mt-Nd3,mt-Nd5,mt-cox1,mt-cox2,mt-ATP6, and mt-ATP8* were markedly decreased after cisplatin treatment which was consistent with previous observations [6]. Interestingly, rotenone treatment partially or completely restored the downregulation of these genes in line with a partial

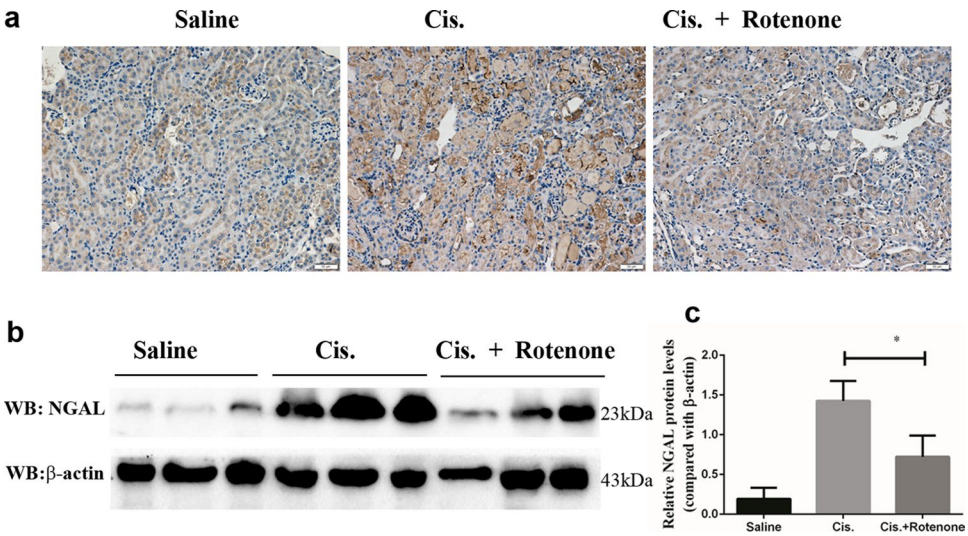

Fig. 2 Rotenone treatment decreased the upregulation of NGAL induced by cisplatin. **a** Representative Immunohistochemistry staining of NGAL in control (saline), cisplatin, and cisplatin + Rotenone groups (original magnification × 200). **b** The protein levels of NGAL were analyzed by Western blotting, and β-actin was used as loading control. **c** Densitometry analysis of the western blots of NGAL. All experiments were duplicated for three times. Data were expressed as means ± S.D. *$P < 0.01$, $n = 8$

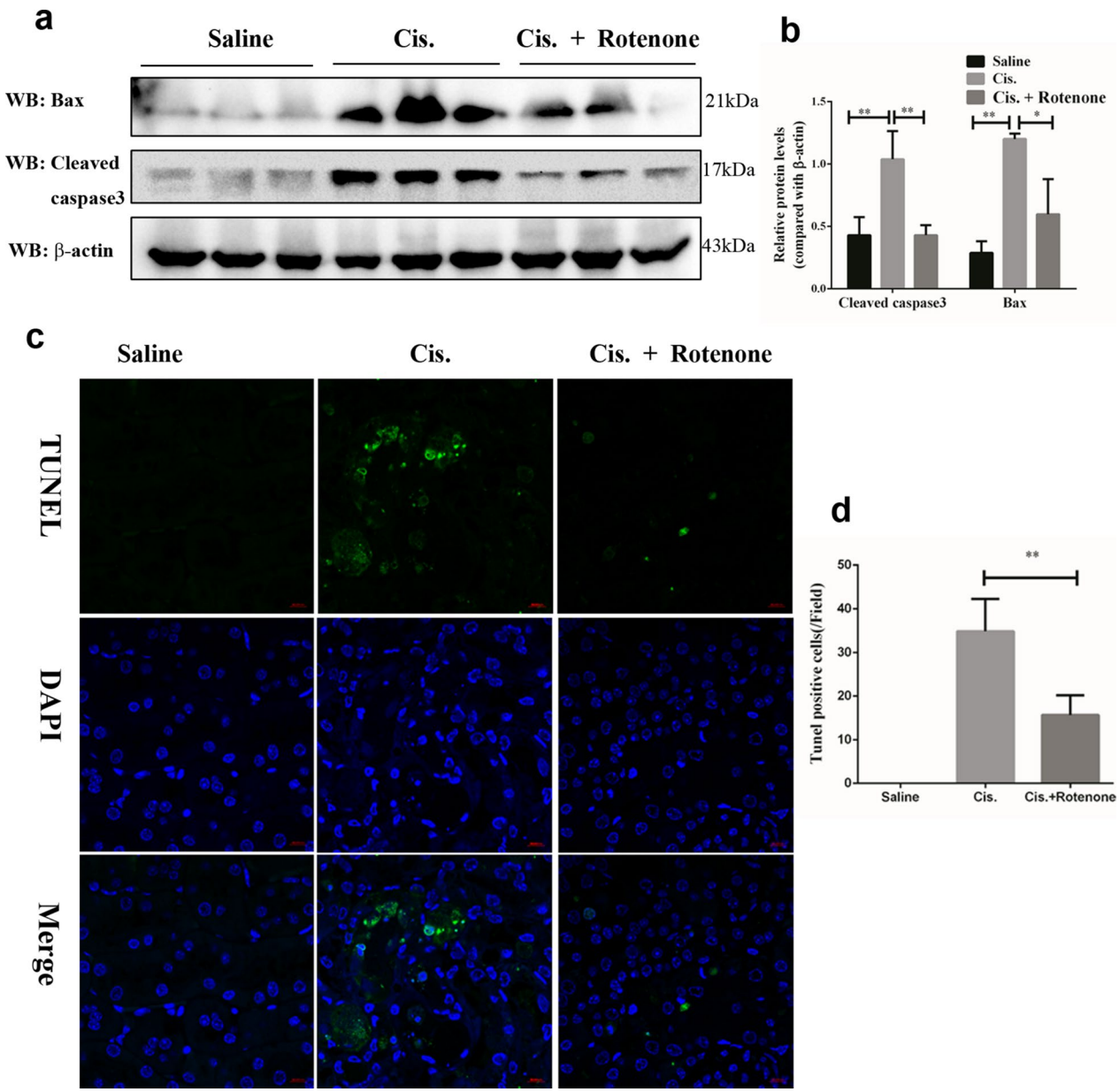

Fig. 3 Rotenone treatment blunted apoptosis in the kidneys of mice treated by cisplatin. **a** Western blotting analysis of Bax and cleaved caspase-3 levels in the kidneys of cisplatin-treated mice with or without rotenone administration. β-actin was used as loading control. **b** Densitometry analysis of the western blots of Bax and cleaved caspase-3. **c** TUNEL staining of kidney slides (original magnification × 630; green: TUNEL; blue: DAPI). **d** The quantification of the number of TUNEL-positive cells. Data were presented as means ± S.D. of 10 random fields from each slide, $n = 8$ in each group. $^{**}P < 0.01$, $^{*}P < 0.05$

recovery of *Tfam* (Mitochondrial transcription factor A) (Fig. 5a). In addition, the protein levels of mitochondrial SOD (SOD2) and ATPB were also analyzed by western blotting. Cisplatin markedly decreased the protein levels of SOD2 and ATPB in the kidneys of cisplatin-treated group, which was significantly restored after rotenone treatment (Fig. 5c, d). Because dysfunctional mitochondria afford to an important source of ROS production, resulting in lipid peroxidation, we examined MDA level using a commercial MDA assay kit. As expected, the markedly increased MDA content in cisplatin-treated kidneys was strikingly reduced by rotenone administration (Fig. 5b). These data demonstrated a potent effect of rotenone treatment on protecting against cisplatin-induced renal mitochondrial injury and oxidative stress.

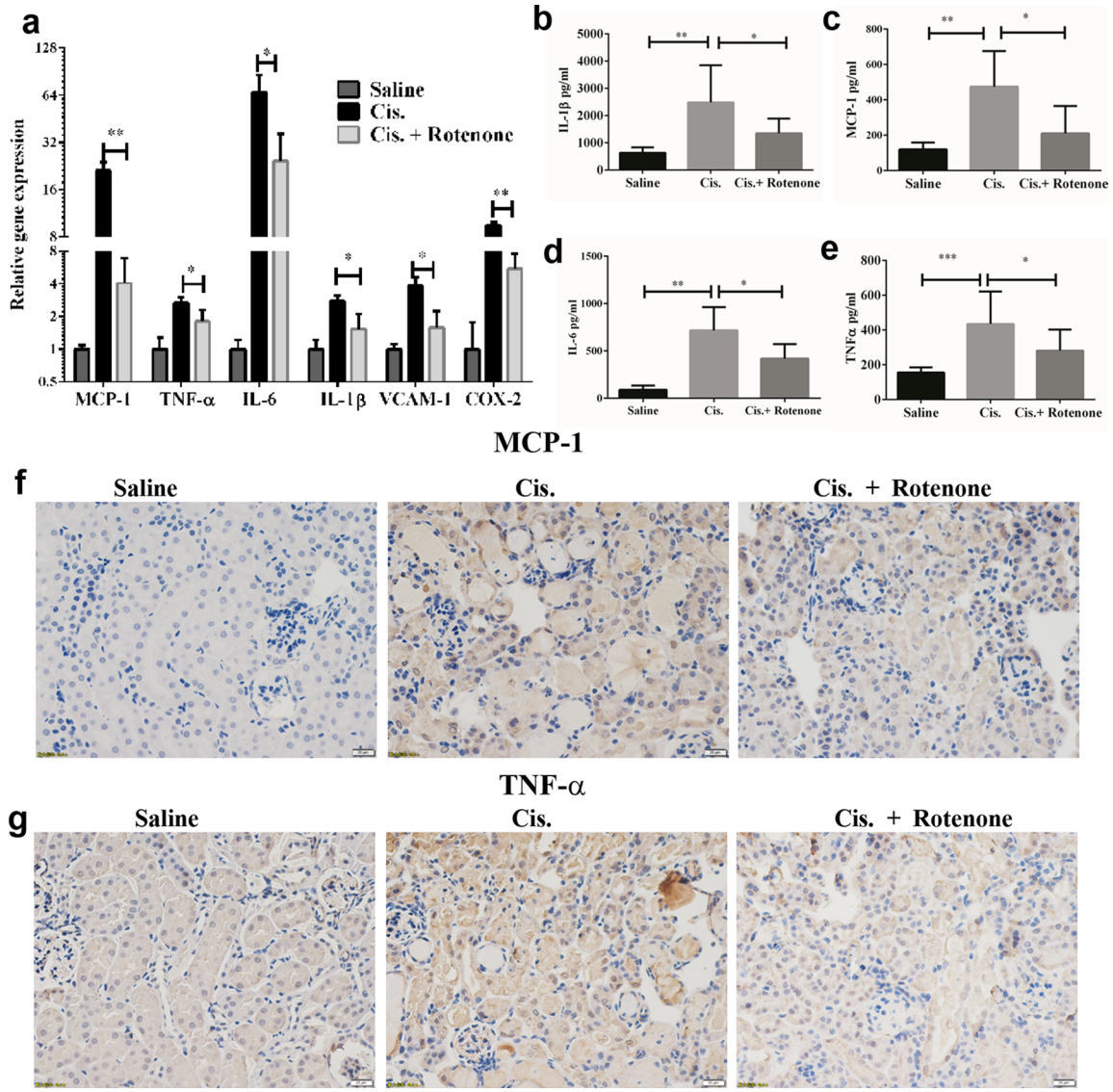

Fig. 4 Rotenone treatment ameliorated inflammatory response induced by cisplatin in mice. **a** The mRNA levels of renal TNF-α, IL-1β, IL-6, MCP-1, VCAM-1, and COX-2 were analyzed by qRT-PCR. **b–e** Enzyme linked immunosorbent assay analysis of circulating IL-1β (**b**), MCP-1(**c**), IL-6 (**d**), and TNF-α (**e**). Data were expressed as means ± S.D, $n = 8$ in each group. Representative Immunohistochemistry staining of MCP-1 (**f**) and TNF-α (**g**) in control (saline), cisplatin, and cisplatin + rotenone groups (Original magnification × 400). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

Rotenone protected against cisplatin-induced renal tubular cell mitochondrial dysfunction and oxidative stress in vitro

We further investigated whether rotenone could directly protect against cisplatin-induced mitochondrial dysfunction and oxidative stress in renal tubular cells in vitro. A cell viability study was performed in cultured mPTCs with rotenone treatment at increasing concentrations from 10 nM to 40 μM for 24 h using a CCK8 assay. The results showed that rotenone at a concentration more than 100 nM decreased the cell viability significantly (Fig. 6a). Thus, we used the concentration of rotenone from 10 nM to 40 nM in following experiments and measured mitochondrial membrane potential (mmp), reactive oxygen species (ROS) production, MDA content, and protein levels of ATPB to evaluate mitochondrial dysfunction and oxidative stress induced by cisplatin in cultured mPTCs. The results showed that cisplatin markedly reduced the mmp, which was significantly blunted by rotenone treatment in a dose-dependent manner without significant difference between 20 and 40 nM treatments (Fig. 6b, c). In the meantime, cisplatin-induced ROS over production in mPTCs was also significantly blocked by rotenone (Fig. 6d, e). Additionally, the reduced protein levels

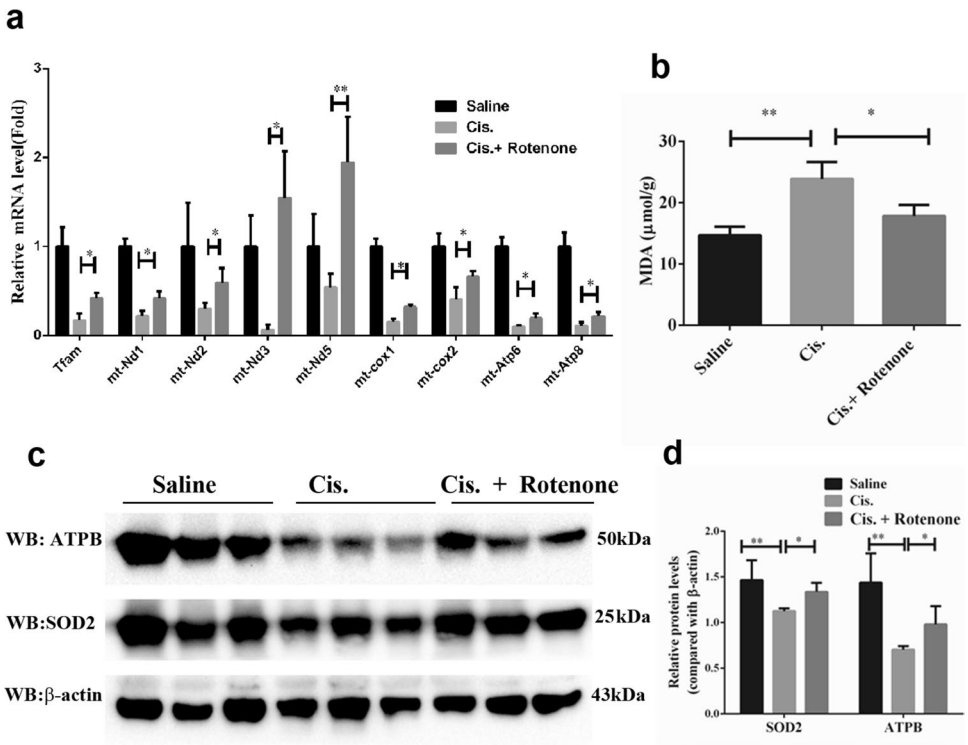

Fig. 5 Rotenone treatment attenuated mitochondrial dysfunction and oxidative stress induced by cisplatin in mice. **a** The mRNA levels of genes encoded by the mitochondrial genome were analyzed by qRT-PCR. **b** The level of MDA in cisplatin-treated mouse kidneys was measured using a commercial kit. **c** Western blotting analysis of ATPB and SOD2 levels in the kidneys of cisplatin-treated mice with or without rotenone treatment. **d** Densitometry analysis of the Western blots of ATPB and SOD2. Data were expressed as means ± S.D. $^{*}P < 0.05$, $^{**}P < 0.01$, $n = 8$

of ATPB were also significantly restored by rotenone in a dose-dependent manner (Fig. 6f, g). Furthermore, we evaluated whether azoxystrobin, an inhibitor of mitochondrial complex III, could inhibit cisplatin-induced ROS production. Our results showed that azoxystrobin was also able to inhibit cisplatin-induced ROS production in vitro (Fig. 8a, b). All of these results demonstrated a direct role of mitochondrial activity inhibition in protecting against cisplatin-induced renal tubular cell mitochondrial dysfunction and oxidative stress in vitro.

Rotenone protected against renal tubular cell apoptosis in vitro induced by cisplatin

In this study, the apoptotic response induced by cisplatin in mPTCs was detected by flow cytometry. As shown in Fig. 7a, b, cisplatin treatment resulted in remarkable mPTC apoptosis, which was markedly inhibited by rotenone treatment in a dose-dependent manner (Fig. 7a, b). In line with the protective effect on cell apoptosis, rotenone treatment decreased the protein levels of cleaved caspase-3 significantly in cisplatin-treated mPTCs (Fig. 7c, d). TUNEL staining further confirmed rotenone effect in antagonizing cisplatin-induced tubular cell apoptosis (Fig. 7e, f). Additionally, we also found that mitochondrial complex III inhibitor azoxystrobin treatment was also able to inhibit cisplatin-induced apoptosis in vitro (Fig. 8c, d). These results demonstrated that rotenone treatment protected against renal tubular cell apoptosis induced by cisplatin.

Discussion

Increasing evidence shows that mitochondrial dysfunction occurs in AKI of various etiologies and contributes to acute renal failure induced by ischemic or toxic [21–23]. Renal tubule cells are reported to be the main target of cisplatin toxicity because these cells are pretty rich in mitochondria [4, 24]. Mitochondrial dysfunction results in the reduction of ATP content and overproduction of ROS. Imbalance between the production and removal of ROS results in oxidative damage, provoking modifications in amino acids, proteins, and lipids, which contributes to cell injury induced by cisplatin [15]. Antioxidants such as melatonin, selenium, vitamin E, vitamin C, quercetin, and so on have been investigated and showed protective effect in different kidney disease models including cisplatin nephrotoxicity [2, 15, 25]. However, the clinical efficacy of these antioxidant agents is still debatable in human patients with cisplatin nephrotoxicity and other types of AKI.

In fact, the critical molecular mechanisms of ROS over production induced by cisplatin and its detrimental effect on renal tubular cell injury remain not fully understood. In order to further clarify the role of mitochondrial dysfunction-derived oxidants on cisplatin-induced kidney injury, we used rotenone, a specific mitochondrial electron transport inhibitor in this study. Rotenone at a high dose and/or long-term treatment was usually used to induce mitochondrial dysfunction disease models such as Parkinson's disease [26, 27]. However, accumulating evidence also indicated

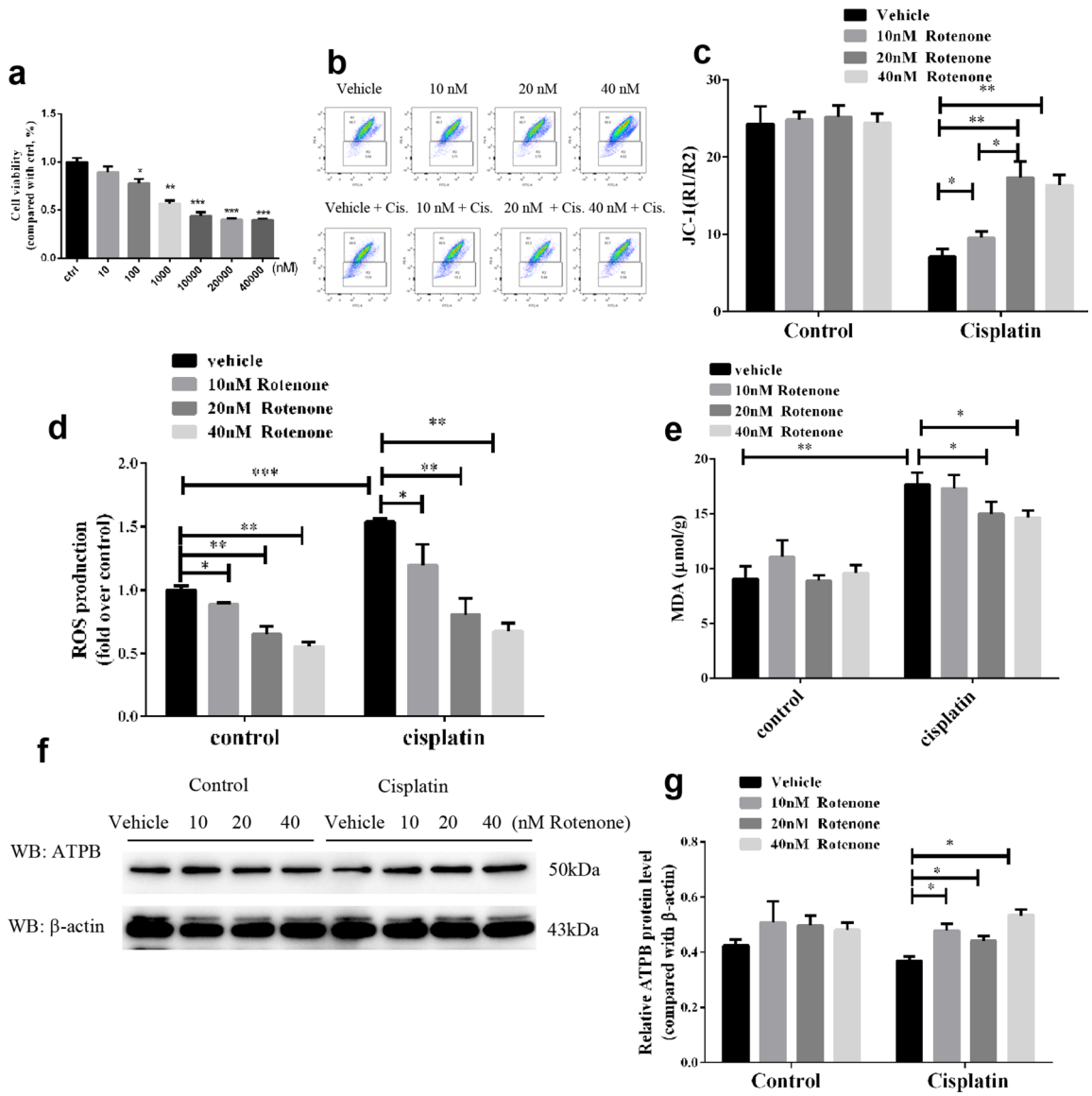

Fig. 6 Rotenone protected against cisplatin-induced renal tubular cell mitochondrial dysfunction and oxidative stress in vitro. **a** Cell viability was analyzed by CCK8 assay after treatment with rotenone for 24 h at increasing concentrations ranging from 10 nM to 40μM. **b** MMP was monitored using MMP-sensitive fluorescent dye JC-1 and analyzed by flow cytometry. **c** Statistical analysis of MMP. **d** ROS production was analyzed by flow cytometry. **e** Oxidative stress marker MDA was measured using a commercial kit. **f** Representative Western blots of ATPB. **g** Quantitative analysis of the western blots of ATPB. mPTCs were treated with cisplatin (5.0 μg/ml) and rotenone for 24 h. β-actin was used as loading control. All experiments were duplicated for three times. Data were expressed as means ± S.D. $^{*}P < 0.05$, $^{**}P < 0.01$

that treatment with rotenone at relatively lower dose could be protective in many disease models including unilateral ureteral obstruction-induced kidney injury, ischemia–reperfusion-induced intestinal mucosal damage, and so on without obvious side effects [16, 17, 28]. In this study, we supposed rotenone treatment at a lower dose could ameliorate the overproduction of ROS, mitochondrial dysfunction, and kidney injury induced by cisplatin. Intriguingly, our results showed that after intraperitoneal injection of the cisplatin into the mice, rotenone treatment at a dose of 200 ppm in food for 3 days significantly protected renal tubular cells against cisplatin-induced apoptosis, inflammation, oxidative stress, and mitochondrial abnormality. Additionally, in vitro experiments showed that treatment with rotenone at lower concentration (less than 40 nM) protected against cisplatin-induced renal tubular cell apoptosis, mitochondrial dysfunction, and oxidative stress, which was not entirely consistent with the report from Kruidering et al. [14]. Although they found 20 μM rotenone reduced the ROS levels induced by cisplatin in Porcine Proximal Tubular Cells (PPTC) from

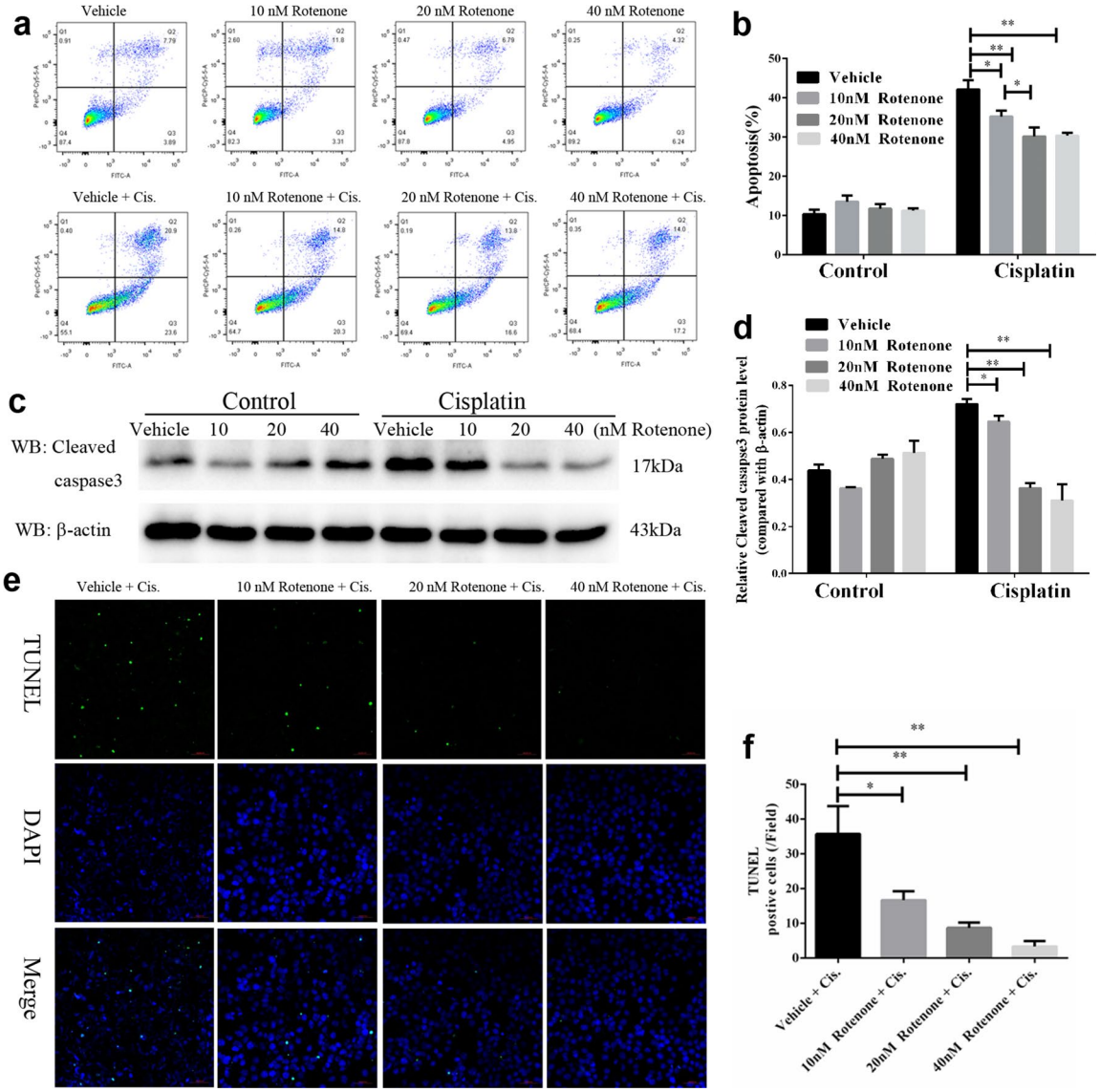

Fig. 7 Rotenone treatment protected against cisplatin-induced mPTC apoptosis. **a** Representative flow cytometry analysis of Annexin V and PI staining. **b** Percentage of apoptotic cells analyzed by flow cytometry. **c** Representative western blots of cleaved caspase-3. **d** Quantitative analysis of the western blots of cleaved caspase-3, mPTCs were treated with cisplatin (5.0 μg/ml) and rotenone for 24 h. β-actin was used as loading control. **e** Representative TUNEL staining (original magnification × 200; green: TUNEL; blue: DAPI). **f** Quantification of the number of TUNEL-positive cells. Data were presented as means ± S.D. of 5 random fields. mPTCs were treated with or without rotenone at different concentration (10, 20, 40 nM) together with cisplatin (5.0 μg/ml) for 24 h. All experiments were duplicated for three times. Data were expressed as means ± S.D. $^{**}P < 0.01$, $^{*}P < 0.05$

664% to almost normal level, 20 μM rotenone treatment did not improve the cisplatin-induced cell apoptosis [14], which might be due to the difference of cell lines. In addition, a mitochondrial complex III inhibitor azoxystrobin also similarly protected renal tubular cells against cisplatin challenge. All these results suggested that suitable inhibition of mitochondrial activity under stress condition could inhibit ROS overproduction and improve mitochondrial dysfunction, which subsequently ameliorated inflammation, cell apoptosis, and kidney injury.

The present study evaluated the protective effects of rotenone in a model of cisplatin-induced AKI. Our results demonstrated that inhibiting the abnormal activity of dysfunctional mitochondria in cisplatin-induced AKI by mitochondrial inhibitors significantly attenuated kidney injury in line with the blockade of apoptosis, oxidative stress, and inflammation. These results suggested a detrimental role of the abnormal mitochondrial activity in cisplatin-induced AKI and targeting mitochondrial complexes may

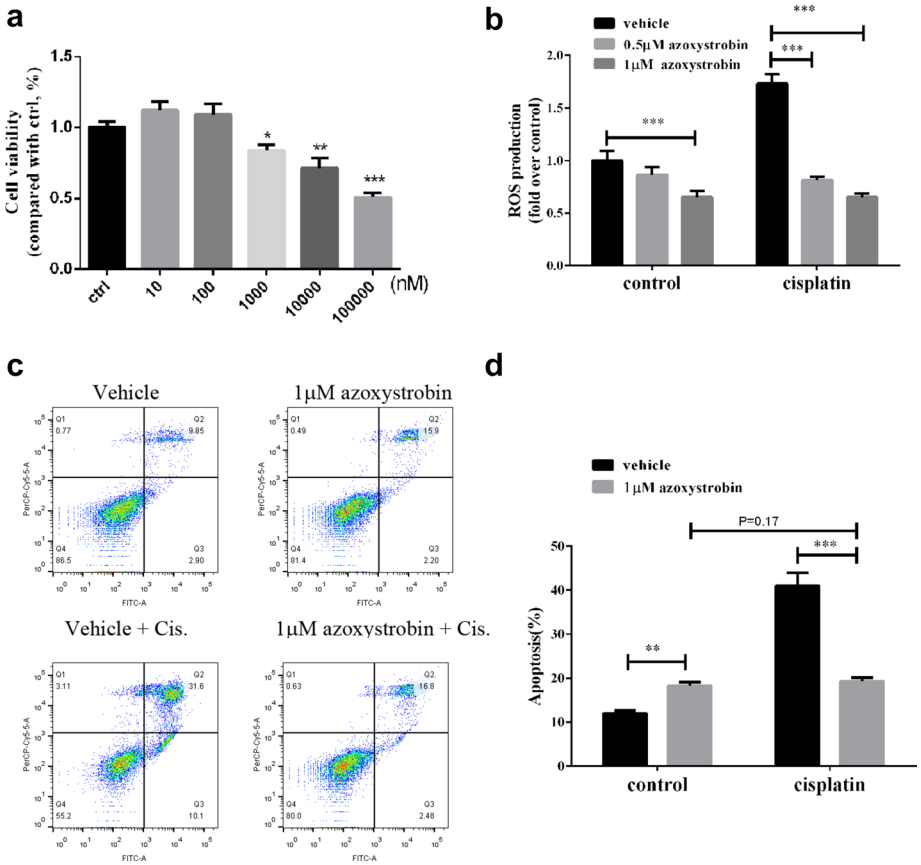

Fig. 8 Azoxystrobin protected against cisplatin-induced renal tubular cell apoptosis and oxidative stress in vitro. **a** Cell viability was analyzed by CCK8 assay after treatment with azoxystrobin for 24 h at increasing concentrations ranging from 10 nM to 100 μM. **b** ROS production was analyzed by flow cytometry. **c** Representative flow cytometry analysis of Annexin V and PI staining. **d** Percentage of apoptotic cells analyzed by flow cytometry. All experiments were duplicated for three times. Data were expressed as means ± S.D. $*P<0.05$, $**P<0.01$, $***P<0.001$

serve as an effective therapeutic strategy for the treatment of cisplatin nephrotoxicity.

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Compliance with Ethical Standards

Conflict of interest The authors state that there is no conflict of interest.

Ethical approval All animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee of Nanjing Medical University (IACUC 14030112-2).

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