TRPA1 promotes cisplatin-induced acute kidney injury by regulating the endoplasmic reticulum stress-mitochondrial damage

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Abstract

Background

Cisplatin (DDP) is a widely used and effective chemotherapeutic agent against cancer. However, nephrotoxicity is one of the most common side effects of DDP, and it can proceed to acute kidney injury (AKI). The aim of this study was to investigate the mechanism of TRPA1 in promoting DDP-induced AKI through modulation of the endoplasmic reticulum stress (ERS)-mitochondrial damage.

Methods

A DDP-induced HK-2 cell model in vitro and mouse model in vivo were established and treated with the TRPA1 antagonist (HC-030031). We also used TRPA1 agonists, and treated with ERS inhibitors or GRP75 inhibitors. Renal function, histopathological changes, apoptosis, ERS and mitochondria-related proteins expression, mitochondrial changes, calcium ion concentration, cell proliferative activity, mitochondrial membrane potential (MMP), ATP, and ROS levels were also evaluated.

Result

DDP increased Scr and BUN levels, caused renal tissue injury and cell apoptosis, decreased ERS-related proteins GRP78, CHOP, and GRP75. The mitochondrial fusion-related proteins OPA1, MFN1, and MFN2, and mitochondrial division-related proteins p-DRP1 and MFF were elevated, DDP lead to mitochondrial dysfunction, and increased calcium ion concentration. In addition, DDP inhibited cell proliferation activity, decreased MMP and ATP levels, and increased ROS levels. In contrast, HC-030031 had protective effects against DDP-induced ERS and mitochondrial dysfunction in vivo and in vitro. Furthermore, TRPA1 agonists promoted mitochondrial dysfunction via mitochondria-associated endoplasmic reticulum membrane. ERS inhibitors and GRP75 inhibitors increased cell proliferation activity, reduced cell apoptosis, and modulated ERS-mitochondrial damage and calcium overload to improve cell injury.

Conclusion

TRPA1 promotes DDP-induced AKI by regulating the ERS-mitochondrial damage.

INTRODUCTION

Cisplatin (DDP) is one of the most commonly used and effective chemotherapeutic agents for various types of human cancers, such as testicular, ovarian, bladder, small and non-small cell lung cancer, cervical, sarcoma and lymphoma (1). Unfortunately, the clinical application of DDP is severely limited by nephrotoxicity, one of the most common side effects of DDP, with about 20–30% of DDP patients suffering from acute kidney injury (AKI) (2). AKI is characterized by high morbidity and mortality.
However, due to its complex etiology, the pathogenesis of AKI remains unclear. Therefore, understanding the molecular pathways and related mechanisms of DDP nephrotoxicity is essential for finding or developing drugs suitable for DDP combination.

Transient receptor potential ankyrin-1 (TRPA1), a member of the transient receptor potential (TRP) channel family, is a non-selective cation channel capable of sensing environmental stimuli (3, 4). It can be activated by a variety of noxious stimuli, causing Ca\(^{2+}\) inward flow and increasing intracellular Ca\(^{2+}\) concentration, which in turn regulates the occurrence of the corresponding physiological functions or pathological mechanisms (5). Previous studies have shown that TRPA1 channels can be activated by endogenous inflammatory mediators and exogenous noxious stimuli, mediating processes such as inflammation and painful stimulation, which may be important targets for anti-inflammation and analgesia (6, 7). In recent years, studies have pointed out that TRPA1 is engaged in the regulation of cell survival and apoptosis (8–10). Our previous study revealed that DDP can promote TRPA1 expression and activation in renal tubular cells and induce cellular Ca\(^{2+}\) inward flow leading to cellular Ca\(^{2+}\) overload; TRPA1 inhibitor HC-030031 can attenuate DDP-induced inflammatory response in renal tubular cells by inhibiting MAPK/NF-κB signaling pathway (11), and also alleviate DDP-induced renal tubular cell apoptosis by inhibiting Ca\(^{2+}\)-dependent signaling pathway (12). These studies suggested that TRPA1 activation mediates the cytotoxic damage of DDP and playa a facilitative role in DPP-induced AKI. However, the molecular mechanism of TRPA1 regulating apoptosis and injury has not been clarified.

In recent years, the role of endoplasmic reticulum stress (ERS) and mitochondrial functional impairment in DDP nephrotoxicity has been extensively reported (13, 14). However, it is unclear whether TRPA1 activation is associated with DDP-induced endoplasmic reticulum stress and mitochondrial function impairment in renal tubular epithelial cells. The endoplasmic reticulum forms a physical connection with mitochondria through the mitochondria-associated endoplasmic reticulum membrane (MAM), which is essential for mitochondrial calcium homeostasis, lipid and metabolite exchange (15–17). Whether TRPA1, as a Ca\(^{2+}\)-dependent cation channel, is associated with endoplasmic reticulum stress and mitochondrial dysfunction deserves further investigation.

Therefore, this study hypothesized that TRPA1 promotes DDP-induced renal tubular epithelial cell injury by inducing Ca\(^{2+}\) overload-endoplasmic reticulum stress-mitochondrial damage. To verify this hypothesis, this study investigated the role of TRPA1 in regulating calcium homeostasis in the MAM between endoplasmic reticulum (ER) and mitochondria in renal tubular epithelial cells to improve DDP-induced AKI using human renal tubular epithelial (HK-2) cells and animal model of DDP-induced AKI. This study will provide a theoretical basis for the treatment of DDP-induced AKI.

**MATERIALS AND METHODS**

**Animals and experiments design**
Male C57BL/6 mice (8–10 weeks, 20–25 g) were purchased from Dashuo Animal Center (Chengdu, Sichuan, China). The mice were placed on a 12 hour/12 hour day/night cycle, allowed to eat and drink freely and were accommodated for a week. This study was approved by the Animal Ethics Committee of West China Hospital (No.20230328001). Then mice were randomly divided into 3 groups (n = 6): control group, model group (DDP), and model + TRPA1 inhibitor group (DDP + HC-030031). DDP and HC-030031 were purchased from Sigma-Aldrich (Merck, Germany). The model group received a single intraperitoneal injection of DDP (20 mg/kg); the model + TRPA1 inhibitor group received a single intraperitoneal injection of DDP (20 mg/kg) followed by gavage administration of the TRPA1 inhibitor HC-030031 (10 mg/kg/d); the control group was injected with equal amount of saline containing 5% DMSO intraperitoneally. 72 h after DDP injection, blood was collected intraperitoneally to isolate serum. All mice were euthanized with chloral hydrate (3 mL/kg, i.p.). Kidney tissue samples were collected immediately and stored at -80°C. Serum was taken to determine blood urea nitrogen (BUN) and serum creatinine (Scr).

**Cell culture and treatment**

Human renal tubular epithelial (HK-2) cells (CL-0109, Procell, Wuhan, China) were cultured in MEM medium (PM150410, Procell) supplemented with 10% fetal bovine serum (FBS) (P20522, TRAN), 100 U/mL penicillin (Sigma, Germany) and 100 g/mL streptomycin (Sigma, Germany). The medium was incubated at 37°C and 5%. Resuscitated cells were cultured in 1:3 passages, and HK-2 cells were plated in 6-well culture plates at 3×10^5 cells per well.

To study the inhibitory effect of TRPA1 inhibitor on DDP-induced endoplasmic reticulum stress in renal tubular epithelial cells. HK-2 cells were divided into three groups (n = 6): Control, DDP, and DDP + HC-030031 groups. The control group was left untreated; the DDP group was induced with 29.16 µM DDP for 48 h; the DDP + HC-030031 group was induced with both DDP and 30 µM HC-030031 for 48 h.

To study the effects of TRPA1 activation on renal tubular epithelial cell injury and endoplasmic reticulum stress. HK-2 cells were divided into four groups (n = 6): control, TRPA1 agonists, TRPA1 agonists + ERS inhibitor, and TRPA1 agonists + GRP75 inhibitor groups. The control group was left untreated; the TRPA1 agonists group was induced with 100 µM AITC (Sigma-Aldrich, Germany) for 24 h; the TRPA1 agonists + ERS inhibitor group was treated with 1 mM 4-PBA (Sigma-Aldrich, Germany); the TRPA1 agonists + GRP75 inhibitor group was induced with both 100 µM AITC and 1 µM MKT077 (MedChemExpress, USA) for 24 h.

**Hematoxylin-eosin (H&E) staining**

Kidney tissue sections were dewaxed to water and stained with hematoxylin for 20 min. Sections were then placed in warm water at 50°C until a blue color appeared. Then stained with eosin for 5 min, and sealed with neutral gum. Finally, the sections were observed at 100x and 400x using an electron microscope (Motic China Group Co., Ltd, China).

**TUNEL staining**
Renal tissue apoptosis was observed using the Tunel Kit (Roche Group, Switzerland). Kidney tissue sections were dehydrated, embedded, and dewaxed to water. Subsequently, the sections were microwave-repaired using citric acid for 8 min, and washed 3 times with PBS. The sections were then incubated in the dark with fluorescent tunel incubation solution at 37°C for 1 h. After PBS washing, the nuclei were stained with DAPI for 15 min. Finally, the sections were scanned using a digital section scanner (3DHISTECH, Hungary) and analyzed.

Transmission electron microscope (TEM)

The mitochondrial damage and morphological changes of kidney tissue and HK-2 cells were observed using transmission electron microscopy. Samples were prefixed with 3% glutaraldehyde and then refixed with 1% osmium tetroxide; dehydrated with acetone step by step, and embedded with Ep812. Prepared 60 nm sections by an ultramicrotome; stained with uranyl acetate for 10 min, followed by lead citrate for 2 min at room temperature. Finally, JEM-1400FLASH transmission electron microscope (JEOL, Japan) was used for image acquisition.

Immunofluorescence

The expressions of ERS marker proteins GRP78, GRP75, and CHOP in HK-2 cells were detected by immunofluorescence. The rehydrated paraffin sections (4µm) were washed with PBS, anti-CHOP (1:100, PA5-104528, Thermo Fisher Scientific, USA), GRP75 (1:100, ab227215, abcam, UK), and GRP78 (1:200, GB11098, Servicebio, China) were incubated overnight at 4°C, and then stained with FITC-labeled goat anti-rabbit IgG (1:100, GB22303, GB21301, GB22303, Servicebio, China) for 30 min. Nuclei were incubated with DAPI for 10 min at room temperature. Finally, the sections were analyzed by a confocal laser microscope (Olympus Corporation, Japan).

Cell viability assay

The CCK-8 assay was used to detect cell viability. HK-2 cells at logarithmic growth stage were taken and inoculated in 96-well plates at a cell density of 4×10⁴/mL, and incubated at 37°C, 5% CO₂ at constant temperature. The supernatant was discarded, and new medium (100 µL) containing CCK8 reagent (10 µL) (Biosharp, China) was added; the medium was gently shaken several times and incubated at 37°C and 5% CO2 for 2 h. The absorbance value of each well was measured at 450 nm using an enzyme marker (molecular devices, Germany).

Flow cytometry

HK-2 cell apoptosis was evaluated by flow cytometry. HK-2 cells at logarithmic growth stage were adjusted to 3×10⁵/well, then inoculated in 6-well plates, and incubated at 37°C and 5% CO₂ at constant temperature. The supernatant was aspirated and washed with PBS. Digest with trypsin and centrifuge at 250 g for 5 min to obtain cell precipitate. Add PBS wash again and then centrifuge. Then HK-2 cells were resuspended with 500 µL of binding buffer and 5 µL of Annexin V-APC, and then 5 µL PI was added and stained in the dark for 15 min at room temperature. Cell apoptosis was detected by flow cytometry (Beckman, Germany).
Western blotting

The total proteins in kidney tissue and HK-2 cells were extracted by RIPA lysate (Servicebio, China), and the supernatant was centrifuged to determine the protein concentration using the BCA protein quantification kit (No. P0009, Beyotime, China). The proteins were denatured by incubation at 95°C for 15 min, separated by 10% SDS-PAGE gel, and then transferred to polyvinylidene difluoride (PVDF) membranes. PVDF membranes were placed in 5% skim milk diluted with TBST Buffer and incubated for 2 h. Subsequently, PVDF membranes were placed in primary antibody and incubated overnight at 4°C, and then placed in secondary antibody and incubated at room temperature for 2 h. Antibody information was shown in Table 1. After washing with TBST, ECL luminescent solution was added and the bands were scanned by explosion using a chemiluminescent gel imager (Shanghai Tanon Technology Co., Ltd, China).

### Table 1
Information about antibodies for Western blotting.

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<td>Abclonal</td>
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### Ca\(^{2+}\) concentration determination

Fluo-2/AM was used to measure the cellular Ca\(^{2+}\) concentration. HK-2 cells were cultured in 6-well plates in 3×10\(^5\)/well. The cells were washed with PBS and centrifuged at 250 g for 5 min to obtain cell pellets. Incubate with Fluo 2-AM solution at 37°C in the dark for 40 min. Wash twice with PBS, and then resuspend in 300 µL fluo-2/AM diluent for 10 min at 37°C. Finally, cellular Ca\(^{2+}\) concentration was measured by flow cytometry. Kidney tissue was ground with a grinder, the cell suspension was collected;
and added red blood cell lysate for 5 min, washed twice with PBS, and the cell pellet was collected by centrifugation. Then Ca^{2+} concentration was measured after staining with Fluo 2-AM solution.

**JC-1 assay**

JC-1 assay was performed to measure mitochondrial membrane potential. HK-2 cells were inoculated in 6-well plates at a density of 3\times10^5/well, and incubated at 37°C with 5% CO_2. The JC-1 working solution was prepared by mixing 900 µL sterilized deionized water, 2 µL JC-1 (500x), and 100 µL 10× incubation buffer. The cells were then incubated at 37°C with 5% CO_2 for 20 min. After centrifuging the cells at 350 g for 5 min, the supernatant was aspirated and washed twice with 1× incubation buffer. Finally, the cells were resuspended in 500 µL 1× incubation buffer for analysis.

**Mitochondrial ATP detection**

The mitochondrial ATP content of HK-2 cells was determined using the ATP content assay kit (A095-1-1, Nanjing Jiancheng Bioengineering Institute, China). HK-2 cell culture supernatant was separated by centrifugation, and the cell were pelleted and broken to determine the protein concentration. The cells were then heated in a boiling water bath for 10 minutes, and vortexed for 1 minute. After standing for 5 min at room temperature, the absorbance values were measured at 636 nm.

**Intracellular ROS levels determination**

The level of ROS in HK-2 cells was detected according to the instructions of the Human ROS ELISA KIT kit (ZCIBIO Technology Co., Ltd, China), and the OD value of each well was measured at 450 nm.

**Statistical analysis**

SPSS 17.0 statistical software (SPSS Inc., USA) was used for statistical analysis. Data were expressed as Mean ± SD, and the One-Way ANOVA test was used for comparison between multiple sample means, and the LSD test (homogeneity of variance) or Tamhane's T2 test (heterogeneity of variance) was performed. The test results were considered significant when \( p < 0.05 \) for differences between groups.

**RESULTS**

**TRPA1 inhibitor HC-030031 protects DDP-induced AKI in mice**

To investigate whether TRPA1 inhibitor HC-030031 has a protective effect in DDP-induced AKI, we evaluated renal function indicators (Scr and BUN), histopathological changes and apoptosis in the kidney. As shown in Fig. 1A and B, compared with the control group, Scr and BUN levels were significantly elevated in the DDP group (both \( P < 0.001 \)), and the DDP + HC-030031 group significantly reduced the DDP-induced Scr and BUN levels (\( P < 0.001, P < 0.05 \)). H&E staining showed some pathological changes in the DDP group, which were characterized by localized tubular epithelial cell swelling, degenerative necrosis, protein tubular pattern seen in some tubular lumens, a small amount of inflammatory cell
infiltration, and a small amount of fibrous tissue hyperplasia. HC-030031 treatment significantly alleviated the pathological damage of the kidney (Fig. 1C). Subsequently, we evaluated the effect of HC-030031 on cell apoptosis in the kidney tissue of DDP injected mice by TUNEL staining. The results showed that TUNEL-positive cells were not found in normal cells, but were significantly found in the kidney tissues of the DDP group ($P<0.01$). HC-030031 treatment significantly reduced the percentage of positive expression of apoptotic cells ($P<0.01$) (Fig. 1D). Taken together, our results suggest that TRPA1 inhibitor HC-030031 ameliorates DDP-induced kidney function, histopathological damage, and apoptosis in AKI mice.

**TRPA1 inhibitor HC-030031 protects endoplasmic reticulum stress-mitochondrial damage in DP-induced AKI in mice**

To investigate whether TRPA1 expression is associated with ERS and mitochondrial morphological dysfunction during DDP-induced AKI in mice, we examined the expression levels of ERS, mitochondrial morphology (division/fusion) related proteins using western blotting, observed mitochondrial damage and morphological changes by TEM, and measured the intra-mitochondrial calcium ion concentration using the Fluo-2 AM calcium ion probe. As shown in Fig. 2A, the expression levels of ERS-related proteins GRP78, CHOP, and GRP75 were significantly elevated in the DDP group ($P<0.01$, $P<0.001$, $P<0.01$), while they were significantly reduced in the DDP + HC-030031 group ($P<0.05$, $P<0.01$, $P<0.05$). As shown in Fig. 2B, the expression levels of mitochondrial fusion-related proteins OPA1, MFN1, and MFN2 were reduced in the kidneys of mice with DDP-induced AKI ($P<0.01$, $P<0.01$, $P<0.001$), but HC-030031 reversed these levels ($P<0.05$, $P<0.05$, $P<0.01$). As shown in Fig. 2C, the expression levels of mitochondrial division-related proteins p-DRP1 and MFF were increased in the DDP group ($P<0.001$, $P<0.01$), while the expression of the proteins was decreased after HC-030031 treatment (both $P<0.05$). TEM results showed that the mitochondrial morphological structure of epithelial cells in kidney tissue was obviously abnormal in the DDP group; the mitochondria were swollen, cristae were dissolved and broken, matrix particles were reduced; the rough endoplasmic reticulum was expanded in a vesicle-like structure; lipid droplets and autophagy were seen in the cytoplasm. However, HC-030031 improved mitochondrial damage in the kidney (Fig. 2D). In addition, it was confirmed that DDP treatment enhanced intracellular calcium ion concentration, while HC-030031 treatment decreased intracellular calcium ion concentration (both $P<0.05$) (Fig. 2E). The above results suggested that TRPA1 may have the effect of mediating DDP-induced AKI in mice, while inhibition of TRPA1 may protect kidneys from ERS-mitochondrial injury.

**TRPA1 inhibitor ameliorates DDP-induced ERS and mitochondrial damage in renal tubular epithelial cells**

**TRPA1 inhibitor HC-030031 inhibits DDP-induced ERS in renal tubular epithelial cells**
We have previously demonstrated the protective effect of TRPA1 inhibitor HC-030031 on AKI in mice. Then we established a DDP-induced renal tubular epithelial cell injury model at the cellular level to further analyze the mechanism of TRPA1-triggered ERS during DDP-induced renal tubular epithelial cell injury. CCK-8 results showed that HC-030031 treatment increased the DDP-induced decrease in proliferative activity of HK-2 cells ($P < 0.05$) (Fig. 3A). Additionally, HC-030031 treatment significantly attenuated DDP-induced apoptosis in HK-2 cells ($P < 0.05$) (Fig. 3B). Furthermore, we used western blotting to detect the expression levels of ERS marker proteins GRP78, GRP75, and CHOP. The results showed that DDP induced an increase in the expression of GRP78, GRP75, and CHOP ($P < 0.01$, $P < 0.01$, $P < 0.001$), while HC-030031 decreased their expression ($P < 0.05$, $P < 0.05$, $P < 0.01$) (Fig. 3C). In short, HC-030031 had an inhibitory effect on DDP-induced ERS in renal tubular epithelial cells.

**TRPA1 inhibitor HC-030031 ameliorates DDP-induced mitochondrial damage and calcium overload in renal tubular epithelial cells**

Then we investigated the relationship between TRPA1 expression and mitochondrial morphological dysfunction and calcium overload during DDP-induced renal tubular epithelial cell injury. Western blotting was used to detect the expression levels of mitochondrial fusion (OPA1, MFN1, and MFN2) and division (DRP1, p-DRP1, and MFF) proteins. The results indicated that HC-030031 increased the DDP-induced decrease in the expression of mitochondrial fusion proteins (OPA1, MFN1, and MFN2) ($P < 0.05$, $P < 0.001$, $P < 0.05$) (Fig. 4A), and decreased the DDP-induced increase in the expression of mitochondrial division proteins (p-DRP1 and MFF) ($P < 0.001$, $P < 0.05$) (Fig. 4B). This result was consistent with the results of the AKI mouse model. The mitochondrial membrane potential of HK-2 cells was detected using the JC-1 assay. As shown in Fig. 4C, HC-030031 treatment significantly elevated the mitochondrial membrane potential of HK-2 cells compared with the DDP-treated group ($P < 0.001$). In addition, TEM results showed that HC-030031 alleviated DDP-induced mitochondrial morphological damage. There were MAM structures between mitochondria and rough endoplasmic reticulum (RES) in the DDP group, while HC-030031 may reduce the MAM area. (Fig. 4D). Fluo-2 AM calcium probe results showed that calcium ion concentration was increased in the DDP group ($P < 0.001$), while inhibition of TRPA1 decreased the calcium ion concentration ($P < 0.001$) (Fig. 4E). Moreover, the TRPA1 inhibitor HC-030031 also reduced the DDP-induced up-regulation of intracellular ROS levels ($P < 0.05$), and increased DDP-induced down-regulation of mitochondrial ATP ($P < 0.001$) (Fig. 4F, G). In summary, TRPA1 inhibitors had an ameliorative effect on DDP-induced mitochondrial morphological and functional impairment and Ca$^{2+}$ overload in renal tubular epithelial cells.

**TRPA1 promotes mitochondrial dysfunction in renal tubular epithelial cells through MAM**

*TRPA1 agonists promotes renal tubular epithelial cell injury and ERS, while ERS inhibitors and GRP75 inhibitors ameliorate cell injury*
The effects of HC-030031 on ER and mitochondrial damage have been verified previously, respectively. However, whether TRPA1-promoted ERS mediates mitochondrial morphology and dysfunction via endoplasmic reticulum-mitochondrial coupling (MAM) deserves further investigation. The CCK-8 results showed that TRPA1 agonists (AITC) decreased the proliferative activity of HK-2 cells, whereas ERS inhibitor (4-PBA) and GRP75 inhibitor (MKT077) treatment significantly increased the proliferative activity of the cells (both \( P < 0.001 \)) (Fig. 5A). In addition, we evaluated the role of TRPA1 agonists in apoptosis. In Fig. 5B, TRPA1 agonists induced apoptosis in HK-2 cells, which was ameliorated by ERS inhibitor and GRP75 inhibitor (both \( P < 0.01 \)). Furthermore, we detected ERS-tagged proteins GRP78, GRP75, and CHOP expression by western blotting as well as immunofluorescence staining. As shown in Fig. 5C, D, GRP78, GRP75 and CHOP expression were elevated in the TRPA1 agonists group compared with the control group (\( P < 0.001, P < 0.001, P < 0.01 \)); while compared with the TRPA1 agonists group, the expression of GRP78, GRP75 and CHOP was decreased in the TRPA1 agonists and TRPA1 agonists + ERS inhibitor groups (both \( P < 0.05 \)). The results further suggest that TRPA1 damage to renal tubular epithelial cells is associated with triggering ERS.

\[ \text{TRPA1 agonists mediated mitochondrial calcium overload promotes mitochondrial morphological damage and dysfunction in renal tubular epithelial cells, while ERS inhibitors and GRP75 inhibitors ameliorate cell injury} \]

MAM was used to indicate the interaction between the endoplasmic reticulum and the mitochondria. The number and morphological changes of cellular mitochondria and MAM were observed by TEM. The results showed that TRPA1 agonists promoted mitochondrial morphological damage and increased MAM area, while ERS inhibitor and GRP75 inhibitor decreased cellular mitochondrial damage and MAM area (Fig. 6A). Western blotting was used to detect mitochondrial fusion (OPA1, MFN1, and MFN2) and division (DRP1, p-DRP1, and MFF) protein expression. As shown in Fig. 6B, ERS inhibitor and GRP75 inhibitor elevated the TRPA1 agonists-induced decrease in OPA1, MFN1, and MFN2 protein levels. Conversely, ERS inhibitor and GRP75 inhibitor reduced DRP1, p-DRP1, and MFF protein levels elevated by TRPA1 agonists (Fig. 6C). In addition, TRPA1 agonists decreased MMP and mitochondrial ATP (both \( P < 0.001 \)), which were significantly increased by ERS inhibitor and GRP75 inhibitor (\( P < 0.001, P < 0.01, P < 0.05, P < 0.01 \)) (Fig. 6D, E). ERS inhibitor and GRP75 inhibitor also decreased TRPA1 agonists-induced increase in intracellular ROS levels (\( P < 0.01, P < 0.05 \)) (Fig. 6F). By observing Ca\(^{2+}\) changes, it was found that TRPA1 agonists promoted mitochondrial calcium overload, which was significantly ameliorated by ERS inhibitor and GRP75 inhibitor (both \( P < 0.001 \)) (Fig. 6G). The above results confirmed that TRPA1 may induce mitochondrial calcium overload by triggering ERS and up-regulating GRP75 expression, thus promoting mitochondrial morphology and dysfunction.

**DISCUSSION**

Cisplatin, as the most widely used chemotherapeutic drug in clinical practice, has a very good effect on inducing tumor cell death. However, cisplatin is easy to reach cells throughout the body with blood circulation, and is often accompanied by various toxic side effects such as nephrotoxicity, neurotoxicity,
ototoxicity, and drug resistance. The DDP-induced acute kidney injury (AKI), with a high mortality rate, is a serious public health problem. Numerous studies have shown that AKI is closely associated with oxidative stress, immune inflammation, apoptosis, mitochondrial damage, ERS, and other causes (18–20).

TRPA1, a calcium-permeable nonselective cation channel, has been reported to play a key role in various types of pain, and TRPA1 antagonists can reduce or eliminate DDP-induced neuropathic pain (21, 22). Our previous studies have revealed that DDP promotes TRPA1 expression, and TRPA1 promotes DDP-induced renal tubular apoptosis through activation of the calcium-dependent NFAT/p53 signaling pathway (12). In the present study, we demonstrated that DDP can cause renal tissue injury and cell apoptosis, and TRPA1 inhibitor HC-030031 can significantly alleviate kidney dysfunction, histopathological injury and cell apoptosis in mice, thus playing a protective role against kidney injury, which is consistent with the previous studies.

ERS is a protective mechanism for cells, while excessive ERS also induces apoptosis (23, 24). ERS may exacerbate acute kidney injury by up-regulating CHOP expression, activating GRP78, and promoting the Caspase 12 apoptotic pathway (25, 26). Mitochondrial homeostasis is essential for normal renal function, mitochondrial function is impaired and further mediates renal dysfunction in AKI (27). As the most prevalent signal transduction factor in the body, calcium ions play an important role in cell division, growth, and death (28). Studies have reported that the occurrence of both endoplasmic reticulum stress and mitochondrial dysfunction are closely related to intracellular Ca\(^{2+}\) overload (29). ERS is accompanied by disturbed Ca\(^{2+}\) homeostasis. Ca\(^{2+}\) is released from the ER into the cytoplasm when ERS is aggravated, and the increased intracellular Ca\(^{2+}\) concentration and oxidative stress increase the Ca\(^{2+}\) concentration in mitochondria, which leads to a decrease in mitochondrial membrane potential, activation of caspase proteins, and initiation of apoptosis (30). As an important regulatory channel of Ca\(^{2+}\) homeostasis, TRPA1 may have the effect of inducing endoplasmic reticulum stress and mitochondrial dysfunction. Endoplasmic reticulum-mitochondrial coupling (MAM) is an important structure for maintaining endoplasmic reticulum and mitochondrial homeostasis, and endoplasmic reticulum stress can promote mitochondrial morphological and functional damage through GRP75-mediated Ca\(^{2+}\) overload (31). Studies have reported that MAM plays an important role in the maintenance of cellular Ca\(^{2+}\) homeostasis. Cellular Ca\(^{2+}\) overload triggers a cascade reaction of reactive oxygen species (ROS) production, leading to decreased MMP and mitochondrial damage, and MAM promotes renal tubular cell death by mediating massive Ca\(^{2+}\) transport (32, 33). In this study, we investigated the balance of TRPA1 regulation of endoplasmic reticulum and mitochondrial homeostasis by introducing MAM to reveal that TRPA1 mediates the cytotoxic effects of DDP-induced ERS promoting mitochondrial dysfunction. Furthermore, we revealed the protective effects of TRPA1 inhibitors on DDP-induced ERS and mitochondrial morphological dysfunction in renal tubular epithelial cells in vivo and in vitro. We also investigated the mechanism of TRPA1 through inducing ERS to promote renal tubular epithelial cell injury and mitochondrial morphological dysfunction by using TRPA1 agonist combined with ERS inhibitor to induce renal tubular epithelial cells. In addition, to investigate the role of MAM in TRPA1-induced ERS
promoting mitochondrial injury, we used TRPA1 agonist combined with GRP75 inhibitor to intervene in renal tubular epithelial cells. The results demonstrated that TRPA1 promotes mitochondrial morphological dysfunction by inducing endoplasmic reticulum-mitochondrial Ca\(^{2+}\) overload. Briefly, TRPA1 may promote mitochondrial morphology and dysfunction by triggering ERS and up-regulating GRP75 expression to induce mitochondrial calcium overload, whereas TRPA1 inhibitor HC-030031 protects against DDP-induced ERS-mitochondrial damage and reduces calcium overload in AKI. Additionally, although this study showed a pro-apoptotic effect of TRPA1, the key mechanisms by which TRPA1 regulates the ERS-mediated apoptotic pathway remain to be further investigated.

In summary, TRPA1 can promote mitochondrial morphology and dysfunction by inducing ERS to mediate DDP-induced renal tubular epithelial cell injury, and TRPA1 can promote mitochondrial morphology and dysfunction by triggering ERS and inducing mitochondrial calcium overload through up-regulation of GRP75 expression. This study demonstrated the mechanism of TRPA1-induced endoplasmic reticulum stress and mitochondrial damage in promoting DDP-induced renal tubular epithelial cell injury. Furthermore, this study provides an important theoretical basis for revealing the toxic role of TRPA1 in DPP-induced renal tubular epithelial injury and targeting TRPA1 inhibition to improve AKI. We also linked ERS with mitochondrial injury by MAM to explore the core effect of TRPA1-induced ERS promoting mitochondrial morphology and dysfunction in mediating DDP nephrotoxicity, providing a new perspective for the treatment of TRPA1-mediated related cellular injury such as DDP-induced AKI.

Declarations

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Not applicable.

AUTHOR CONTRIBUTIONS

The study design was contributed by Fei Deng, Heping Zhang and Wei Qin. The formal analysis was contributed by J Fei Deng, Wei Yang, and Liangbin Zhao. Writing the original draft was contributed by Fei Deng, Heping Zhang, and Wei zhou. Experiment and data acquisition were contributed by Fei Deng, Heping Zhang, Wei zhou, and Shijie Ma, Yuwei Kang. Writing and editing the manuscript were contributed by Fei Deng, Heping Zhang, Wei zhou and Wei Yang. All the authors approved the final version of the manuscript.

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Availability of data and materials

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Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no conflicts of interest.

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**Figures**
HC-030031 ameliorates DDP-induced kidney function, histopathological damage, and apoptosis in AKI mice. HC-030031 reduced Scr (A) and BUN (B) concentrations in serum. (C) H&E staining was used to observed pathological changes of the kidney. A 4-point scale was used for scoring, with no lesions recorded as 0, slight as 1, mild as 2, moderate as 3, and severe as 4. Renal tubular epithelial degeneration necrosis (green arrows) lymphocytes (red arrows), fibroblasts (yellow arrows), and protein tubular pattern (blue arrows) are shown in the figure. Scale bar = 50 µm. (D) Renal cell apoptosis was observed by TUNEL fluorescence staining. Blue light represents DAPI, green light represents Tunel, red arrows represent normal cells, and yellow arrows represent apoptotic cells. Scale bar = 20 µm. The results were
shown as means ± SD. **P < 0.01, ###P < 0.001, compared with the control group; *P < 0.05, **P < 0.01, ###P < 0.001, compared with the DDP group.

Figure 2

HC-030031 protects endoplasmic reticulum stress-mitochondrial damage in DP-induced AKI in mice. (A) The ERS-related proteins GRP78, CHOP, and GRP75 levels were measured using western blot. (B) The
levels of mitochondrial fusion proteins OPA1, MFN1, and MFN2 were analyzed using western blot. (C) The expression levels of mitochondrial division proteins DRP1, p-DRP1 and MFF were analyzed using western blot. (D) HC-030031 improved mitochondrial damage in the kidney. Mitochondria are represented by red arrows, autophagy is represented by green arrows, lipid droplets are represented by blue arrows, and the rough endoplasmic reticulum is represented by yellow arrows. Scale bar = 2 μm. (E) HC-030031 decreased intracellular calcium ion concentration. The western blot results were exhibited after being normalized to β-actin. The results were shown as means ± SD. ##P < 0.01, ###P < 0.001, compared with the control group; *P < 0.05, **P < 0.01, compared with the DDP group.

**Figure 3**

**HC-030031 inhibits DDP-induced ERS in renal tubular epithelial cells.** (A) Cell proliferation activity was detected by CCK8 assay. (B) The apoptotic of the HK-2 cells was determined by flow cytometry. (C) The ERS-related proteins GRP78, CHOP, and GRP75 levels in HK-2 cells were measured using western blot. The results were shown as means ± SD. ##P < 0.01, ###P < 0.001, compared with the control group; *P < 0.05, **P < 0.01, compared with the DDP group.
HC-030031 ameliorates DDP-induced mitochondrial damage and calcium overload in renal tubular epithelial cells. (A) The levels of mitochondrial fusion proteins OPA1, MFN1, and MFN2 were analyzed using western blot. (B) The expression levels of mitochondrial division proteins DRP1, p-DRP1 and MFF were analyzed using western blot. (C) Mitochondrial membrane potential of cell was detected by JC-1 assay. (D) The morphological changes of mitochondria were observed by TEM. Mi, mitochondria; RER,
rough endoplasmic reticulum; the black arrows represent MAM. Scale bar = 200 nm. (E) Fluo-2/AM was used to measure Ca$^{2+}$ concentration in HK-2 cells. (F) HC-030031 reduced the DDP-induced up-regulation of intracellular ROS levels. (G) HC-030031 increased DDP-induced down-regulation of mitochondrial ATP. The results were shown as means ± SD. $^\# P < 0.05$, $^\## P < 0.01$, $^{###} P < 0.001$, compared with the control group; $^* P < 0.05$, $^{***} P < 0.001$, compared with the DDP group.

**Figure 5**
TRPA1 agonists promote renal tubular epithelial cell injury and ERS, while ERS inhibitors and GRP75 inhibitors ameliorate cell injury. (A) Cell proliferation activity of HK-2 cell was detected by CCK8 assay. (B) The apoptotic of the HK-2 cells was determined by flow cytometry. (C) ERS-related proteins GRP78, GRP75 and CHOP in HK-2 cells were detected by immunofluorescence staining. Scale bar = 20 µm. (D) The ERS-related proteins GRP78, CHOP, and GRP75 levels in HK-2 cells were measured using western blot. The results were shown as means ± SD. ##P < 0.01, ###P < 0.001, compared with the control group; *P < 0.05, **P < 0.01, ***P < 0.001, compared with the DDP group.
TRPA1 agonists promote mitochondrial morphological damage and dysfunction in renal tubular epithelial cells, while ERS inhibitors and GRP75 inhibitors ameliorate cell injury. (A) The morphological changes of mitochondria were observed by TEM. Mi, mitochondria; RER, rough endoplasmic reticulum; the black arrows represent MAM. Scale bar = 200 nm. (B) The levels of mitochondrial fusion proteins OPA1, MFN1, and MFN2 were analyzed using western blot. (C) The expression levels of mitochondrial
division proteins DRP1, p-DRP1 and MFF were analyzed using western blot. (D) Mitochondrial membrane potential of HK-2 cell was detected by JC-1 assay. (E) ERS inhibitors and GRP75 inhibitors increased DDP-induced down-regulation of mitochondrial ATP. (F) ERS inhibitors and GRP75 inhibitors reduced the DDP-induced up-regulation of intracellular ROS levels. (G) ERS inhibitors and GRP75 inhibitors reduced the DDP-induced up-regulation of \( \text{Ca}^{2+} \) concentration in HK-2 cells. The results were shown as means ± SD. ## \( P < 0.01 \), ### \( P < 0.001 \), compared with the control group; * \( P < 0.05 \), ** \( P < 0.01 \), *** \( P < 0.001 \), compared with the DDP group.