Sirtuin 3 deficiency promotes acute kidney injury induced by sepsis via mitochondrial dysfunction and apoptosis

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Objective(s): To explore the regulation mechanism of Sirtuin 3 (SIRT3) on the mitochondrial function and apoptosis of acute kidney injury (AKI) in septic mice.

Materials and Methods: The sepsis-induced AKI model was constructed in the wild-type and SIRT3 knockout (KO) mice, and the levels of serum creatinine (Scr) and plasma kidney injury molecule 1 (pKIM-1) in mice were detected by ELISA. The mitochondrial damage of kidney tubular epithelial cells (KTEC) was observed by electron microscopy, the apoptosis of KTEC was detected by TUNEL assay, and the mRNA levels of SIRT3, Bax, Caspase-3, and Bcl-2 were detected by RT-qPCR.

Results: SIRT3 KO caused increased expression of Scr, pKIM-1, and inducible nitric oxide synthase protein in the kidneys of septic mice, and increased the levels of superoxide dismutase, catalase, and mitochondrial complex enzymes I/II/III/IV. SIRT3 deficiency exacerbated histopathological and mitochondrial damage to the proximal tubules of the kidney. In addition, SIRT3 KO resulted in a significantly increased apoptosis of KTEC, increased the mRNA levels of Bax and Caspase-3, and decreased the mRNA levels of Bcl-2.

Conclusion: Our study suggests that SIRT3 deficiency promotes sepsis-induced AKI via increased oxidative stress, mitochondrial dysfunction, and inducing apoptosis.

Keywords: Sirtuin 3, Sepsis, Mitochondria, Apoptosis

Introduction

Acute kidney injury (AKI) is characterized by a sudden decrease in glomerular filtration rate, which can be caused by various causes such as renal ischemia, nephrotoxic drugs, sepsis, and urinary tract obstruction (1). The main clinical manifestations of AKI are increased serum creatinine (Scr) concentration and oliguria, the treatment measures include symptomatic treatment for the cause, protection of kidney function, prevention and management of complications, etc (2). The studies indicated that the incidence of AKI in patients with sepsis is 35–50%, and the mortality rate is as high as 35%, and the 90-day mortality rate increases with the severity of the disease (3, 4). Some surviving patients will inevitably develop end-stage kidney disease, threatening the life and health of all human beings.

Mitochondrial injury is one of the pathophysiology of AKI, and silent signal pathways of mitochondrial protein homeostasis play an important role in mitochondrial production, division, fusion, and apoptosis (5). Sirtuins (SIRTs) are composed of approximately 270 amino acid residues, and which have two different domains and a crack between the domains, containing the binding site of nicotinamide adenine dinucleotide, binding nicotinamide adenine dinucleotide after a catalytic reaction (6). Studies found that the SIRT family has seven subtypes, and the difference in N-terminal and C-terminal structures determines the different positioning and function of each subtype in each subcellular structure: SIRT1, SIRT6, and SIRT7 exist in the nucleus, SIRT2 is found in the cytoplasm and nucleus, while SIRT3, SIRT4, and SIRT5 are mainly found in the mitochondria (7, 8). SIRT3, SIRT4, and SIRT5 are responsible for the regulation of protein acetylation (9). Therefore, SIRT3 may have potential value in the mechanism of the occurrence and development of AKI.

In the present study, SIRT3 knockout (KO) mice were used to construct a sepsis model. The present study aims to further study the regulation mechanism of SIRT3 on the mitochondrial function and apoptosis of kidney tubular epithelial cells (KTEC) in septic mice and to provide a new therapeutic target for AKI induced by sepsis.

Materials and Methods

Animals and grouping

SIRT3 KO C57BL6 mice were purchased from Jackson Laboratory (Farmington, Connecticut, USA), male, 6–8 weeks old. Wild-type (WT) C57BL6 mice were purchased from the Experimental Animal Center of Zhejiang University (Hangzhou, China). We kept all mice in a standard environment, keeping light and darkness for 12 hr each, during which the mice were free to eat and drink. The mice were randomly divided into 4 groups (n=10): WT-Sham group, KO-Sham group, WT-CLP group, and KO-CLP group. According to a previous protocol, we used the CLP method to construct a model of sepsis (10). In short, we anesthetized mice with 1.5% pentobarbital (0.1 ml/20 g) intraperitoneally, cut the abdominal wall...
at the midline, ligated the distal cecum, and pierced the proximal cecum with a 27 gauge needle and extruded a small amount of feces, sutured the incision after returning to the cecum. The procedures of the Sham group were the same as that of the CLP group, except for ligation and perforation. At 24 hr after CLP, the mice were sacrificed by neck fracture, and the blood and kidneys were taken for subsequent experiments. All experimental protocols involved in this study were approved by the Experimental Animal Ethics Committee of Ningbo University, and all steps were carried out in accordance with the experimental protocols and standard procedures.

**H&E staining**

We fixed part of the right kidney in 4% paraformaldehyde for 24 hr, washed twice with PBS, and embedded in paraffin after dehydration of conventional tissue. We used a microtome to cut the paraffin block into 2–3 μm sections, and performed H&E staining after routine deparaffinization and removal, and observed the pathological damage under a common optical microscope. Quantitative scores of kidney tubular epithelial histopathological damage: normal, 0; 0–25%, 1; 25–50%, 2; 50–75%, 3; >75%, 4. Specific criteria: kidney tubular shedding and atrophy, cell edema and rupture, basement membrane exfoliation, interstitial inflammatory cell infiltration, and vacuolation (11).

**Immunohistochemistry**

We cut the paraffin block into 2–3 μm sections, after dehydration and clearing, the slices were put in the incubator for 30 min, then taken out and cooled for 15 min. We added the primary antibody [SIRT3 (R&D Co., Shanghai, China)] or inducible nitric oxide synthase (iNOS, R&D Co., Shanghai, China) according to the instructions, counterstained with the ABC kit, and observed under an optical microscope.

**Transmission electron microscopy**

We fixed the kidney cortex with 4% paraformaldehyde and 0.02% picric acid and fixed it again with 1% tetroxide and 1.5% potassium ferrocyanide. We dehydrated the treated kidney and embedded it in resin, and used Accu-Edge to cut the kidney into ultra-thin sections. After staining the sections with lead citrate and 0.02% picric acid and fixed it again with 1% tetroxide and 1.5% potassium ferrocyanide, we observed the sections on a JEM 1400 electron microscope. We observed inflammatory cell infiltration, and vacuolation (11).

**Anti-oxidant enzyme detection**

The levels of superoxide dismutase (SOD) (Abcam Co., Beijing, China) and catalase (CAT) activity (Solarbio Co., Beijing, China) were detected according to the manufacturer’s instructions. The fresh kidney tissue (0.2 g) was taken, ground on ice, centrifuged at 15000 r/min for 15 min, and the supernatant was taken for determination of antioxidant enzymes. A reaction system with 3 ml (0.3% H$_2$O$_2$, 1 ml, H$_2$O 1.95 ml) was added, and 0.05 ml enzyme solution was added to start the reaction system. The OD reduction rate at a wavelength of 240 nm was measured, and SOD and CAT activities were calculated according to the rate of change of absorbance.

**Mitochondrial complex enzyme detection**

We took the kidney cortex, routinely homogenized on ice, centrifuged, added a disposable mitochondrial extraction reagent, homogenized, centrifuged, removed the supernatant, added lysed enzymes, mixed it, and allowed it to stand on ice for 15 min. We used spectrophotometry to measure the activity of mitochondrial enzyme complexes.

**Statistical analysis**

All data were expressed as mean±SEM or percentage. We used Prism 6.02 for mapping, the one-way ANOVA method for comparison between multiple groups, and Tukey's post hoc test. We set $P<0.05$ to be considered statistically significant.

**Results**

**The protective effect of SIRT3 on CLP-induced AKI**

To confirm the protective effect of SIRT3 on CLP-induced AKI, we detected the protein and mRNA expression of SIRT3 in kidneys. Our immunohistochemistry results showed that SIRT3 protein was highly expressed in the kidneys of the WT-Sham group, and CLP caused SIRT3 protein to be significantly reduced (Figures 1A and B). Consistent with the above results, our RT-qPCR results
showed that the mRNA level of SIRT3 was high in the WT-Sham group, compared with that, the mRNA level of SIRT3 was significantly decreased in the WT-CLP group (Figure 1C). Furthermore, we used the ELISA method to detect the levels of Scr and pKIM-1. We found that compared with the WT-Sham group, CLP caused Scr and pKIM-1 levels to significantly increase, and these two indicators were more significantly elevated in KO-CLP mice (Figures 1D and E). The above results preliminarily confirmed the protective effect of SIRT3 on septic AKI, and our pathological analysis results also confirmed this. We found that in the WT-CLP group, KTEC were shed, the glomerular capsule was ruptured, and the renal interstitium was scattered in the inflammatory cells. In the KO-CLP group, KTEC were extensive and the glomeruli were completely destroyed, and a large number of inflammatory cells filled in the interstitium (Figure 1F). Compared with the WT-CLP group, the kidney tissue injury score of the KO-CLP group was significantly increased (Figure 1G). Our results suggested that SIRT3 had a protective effect on CLP-induced AKI.

**The effect of SIRT3 on CLP-induced oxidative stress**

As an oxidase inducer, iNOS can activate and up-regulate various oxidases to inhibit oxidative stress (12). To investigate the effect of SIRT3 on oxidative stress during sepsis-induced AKI, we used immunohistochemistry to detect iNOS protein expression. We found that iNOS protein was both expressed at low levels in WT-Sham and KO-Sham groups, and CLP caused the expression of iNOS protein to significantly increase. Moreover, compared with the WT-CLP group, SIRT3 deficiency resulted in more increased expression of iNOS protein (Figures 2A and B). Moreover, we detected the expressions of antioxidant enzymes SOD and CAT in the kidneys. We found that the levels of antioxidant enzymes SOD and CAT in the WT-CLP group were significantly downregulated compared with the WT-Sham group. However, in the KO-CLP group, SOD and CAT levels were reduced more (Figures 2C and D). These results indicated that loss of SIRT3 activated oxidative stress in septic mice.
SIRT3 deficiency exacerbates mitochondrial dysfunction in CLP-induced AKI

To study the protective effect of SIRT3 on the mitochondrial function of KTEC in CLP-induced AKI, we observed the mitochondrial structure of KTEC by projection electron microscopy. We found that the mitochondrial structure of KTEC was complete and regular in both WT-Sham and KO-Sham groups. However, in the WT-CLP group, the mitochondria were swollen, irregular in structure, and the edges were blurred. In the KO-CLP group, the mitochondrial structure of KTEC was disordered and sparse, making it difficult to distinguish the edges (Figure 3A). Using morphometric analysis, we found that the average mitochondrial density of proximal tubular epithelial cells was significantly reduced in both WT-CLP and KO-CLP groups. Meanwhile, SIRT3 deficiency caused the average mitochondrial density to decrease more (Figure 3B). Also, we detected the levels of mitochondrial complex enzymes I/II/III/IV in the kidney. We found that mitochondrial complex enzymes I/II/III/IV were expressed at high levels in both WT-Sham and KO-Sham groups, and CLP caused

**Figure 3.** Protective effect of SIRT3 on mitochondrial function in CLP-induced AKI. (A) Effect of SIRT3 deficiency on the mitochondrial structure. (B) Quantitative determination of mean mitochondrial density (μm3) in proximal tubular cells. (C) Expression levels of complex I in the kidney. (D) Expression levels of complex II in the kidney. (E) Expression levels of complex III in the kidney. (F) Expression levels of complex IV in the kidney. SIRT3, Sirtuin3; CLP, cecal ligation and perforation; AKI, acute kidney injury. Compared with WT-Sham group, **P<0.01, ***P<0.001; compared with KO-Sham group,** P<0.01, ***P<0.001; compared with WT-CLP group, #P<0.05, ##P<0.01
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these indicators to significantly reduce (Figures 3C-F). Furthermore, compared with the WT-CLP group, the mitochondrial complex enzymes I/II/III/IV of the KO-CLP group decreased more significantly (Figures 3C-F). The above results showed that the deficiency of SIRT3 exacerbated the mitochondrial structural disorder and dysfunction of AKI induced by CLP.

Figure 4. SIRT3 deficiency induces apoptosis of kidney tubular epithelial cells. (A) The apoptosis of kidney tissues detected by TUNEL assay. (B) Apoptotic rate of kidney in each group. (C) RT-qPCR detection of Bcl-2 mRNA in the kidney. (D) RT-qPCR detection of Bax mRNA in the kidney. (E) RT-qPCR detection of Caspase-3 mRNA in the kidney. SIRT3, Sirtuin3; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling assay. Compared with WT-Sham group, **P<0.01, ***P<0.001; compared with KO-Sham group, **P<0.01, ***P<0.001; compared with WT-CLP group, #P<0.05, ##P<0.01

Effect of SIRT3 on apoptosis in CLP-induced AKI.

In recent years, the role of apoptosis in septic AKI has been a hot research topic (13). To explore the effect of SIRT3 on the apoptosis of KTEC in CLP-induced AKI, we used the TUNEL method to detect the apoptosis of the kidney. We found that the number of apoptotic cells was very few in both WT-Sham and KO-Sham groups, but the number of apoptotic cells caused by CLP increased significantly (Figures 3C-F). The above results showed that the deficiency of SIRT3 exacerbated the mitochondrial structural disorder and dysfunction of AKI induced by CLP.

SOD and CAT are involved in the redox reaction in the human body, which can remove harmful metabolic substances produced after peroxidation and prevent lipid peroxidation (14). ROS are produced in the mitochondria of cells and are important biomarkers to judge the generation of oxygen free radicals and tissue damage (15). Under physiological conditions, ROS can regulate various physiological activities, such as cell homeostasis, cell division, and differentiation. However, under special circumstances, due to long-term ischemia, hypoxia, or toxic effects of drugs, the respiratory chain

Discussion

Sepsis is a common cause of AKI (1). In adults, sepsis accounts for 26%–50% of AKI in developed countries and accounts for 7%–10% of AKI related to primary kidney disease (3). Animal experiments showed that SIRT3 alleviates the pathological damage of the kidney and even prolongs the survival period (8). In the septic mice model induced by CLP, the generation of reactive oxygen species (ROS) is increased in kidney tissues of SIRT3 gene knockout mice, and SIRT3 reduces KTEC damage and apoptosis through the inflammatory response signaling pathway of NOD-like receptor family 3 inflammatory bodies, interleukin-1β and interleukin-12, thereby improving kidney function (11).

In the present study, to investigate the protective effect of SIRT3 on CLP-induced AKI, we used immunohistochemistry and RT-qPCR to detect the expressions of SIRT3 protein and mRNA in the kidneys. We found that in the WT-CLP group, the SIRT3 protein and mRNA were expressed at lower levels in the kidneys, and the levels of Scr and pKIM-1 were significantly increased. However, our study showed that SIRT3 knockout aggravated kidney pathological damage, these results confirmed the protective effects of SIRT3 on AKI induced by CLP.
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Our study indicates that SIRT3 deficiency promotes CLP-induced AKI via increasing oxidative stress, inducing mitochondrial dysfunction and apoptosis. There is no effective early treatment method for septic AKI, but SIRT3 as a mitochondrial protein is expected to become an effective treatment target.

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**Conflicts of Interest**

The authors declare that they have no conflicts of interest.

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