NEC-1 ATTENUATES MITOCHONDRIAL DAMAGE IN THE RENAL TUBULAR EPITHELIAL CELLS OF MICE WITH URINARY SEPSIS BY UPREGULATING PGC1-A TRANSCRIPTION

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ABSTRACT

Objective: *To investigate the effect and mechanism of Nec-1 on mitochondrial damage in the renal tubular epithelial cells of septic mice by upregulating the transcription of peroxisome proliferator-activated receptor γ costimulatory factor (PGC1-α).*

Methods: *A total of 18 C57BL/6 mice were randomly and equally divided into a normal control group, an LPS group and an LPS+Nec-1 group. The mice in the normal control group were injected with 10 mL·kg-1 sterile water; the mice in the LPS group were injected with 10 mL·kg-1 LPS intraperitoneally; and the mice in the LPS+Nec-1 group were injected with 1.65 mL·kg-1 NEC followed by 10 mL·kg-1 LPS. After 18 hours, blood was collected from the inferior vena cava and both kidneys, and the levels of serum creatinine, urinary nitrogen, PGC1-α protein and mRNA expression were compared. The HK-2 cells were randomly divided into a normal control group, an LPS group and an LPS+Nec-1 group after conventional culture and passage. The cells in the normal control group were treated with 10 μ g·ml-1 LPS for 18 h, the cells in the LPS+Nec-1 group were pre-treated with 50 μg·ml-1 Nec-1 for 6 h, and then treated with 10 μg·ml-1 LPS for 18 h. The morphological changes of mitochondria were observed.*

Results: *Compared with the normal control group, the levels of serum creatinine and urinary nitrogen in the mice in the LPS group increased significantly (P<0.05), while the levels of serum creatinine and urinary nitrogen in the LPS+Nec-1 group decreased significantly compared with the LPS group (P<0.05). The epithelial structure of the renal tubules in the normal control mice did not change significantly. The number of mitochondria in the renal tubule epithelial cells of mice in the LPS group decreased significantly, and mitochondrial swelling and vacuolation was also observed. In addition, no mitochondrial crests were observed. The degree of mitochondrial swelling in the renal tubular epithelial cells of the mice in the LPS+Nec-1 group was minor: the number of mitochondria did not change significantly and no mitochondrial vacuolation or mitochondrial crest disappearance was observed. Compared with the normal control group, the protein and mRNA expression levels of PGC1-α in the renal tissue of mice in the LPS group decreased significantly (P<0.05), while the protein and mRNA expression levels of PGC1-α in the renal tissue of mice in the LPS+Nec-1 group significantly increased (P<0.05).*

Conclusion: *Nec-1 can reduce mitochondrial damage in the renal tubular epithelial cells of septic mice, and has a protective renal function, which may be achieved by upregulating PGC1-α transcription.*

Keywords: *Sepsis, Nec-1, PGC1-α, serum creatinine.*

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Introduction

In recent years, the incidence of sepsis, which can lead to multiple organ dysfunction and high mortality rates, has been steadily increasing[(1)]. Relevant statistics showed that in the past decade, the overall incidence rate of sepsis is approximately 5%, while its incidence among hospitalised patients is 30%, with a mortality rate as high as 26%[(2)]. Acute kidney injury can be a complication of sepsis, and once it occurs, patient prognosis can significantly worsen[(3)]. Therefore, exploring the effective prevention and treatment of septic acute kidney injuries has become the mainstream direction for clinical scholars both in China and around the world. Damaged mitochondrial function and reduced regeneration can lead to sepsis-induced acute lung injury. Studies have shown that improving mitochondrial production, restoring mitochondrial function and alleviating the degree of mitochondrial injury is conducive to reversing acute

kidney injury[4]. Peroxisome proliferator-activated receptor Y coactivator 1-α (PGC1-α) may be involved in maintaining normal mitochondrial energy metabolism and production, and a downregulation in its expression can lead to a decrease in mitochondrial metabolism, oxidative phosphorylation and mitochondrial production[5]. Studies related to acute kidney injury have shown that the resulting reduced expression level of PGC1-α can lead to mitochondrial function, structural damage and mitochondrial regeneration disorders[6]. Necrostatin-1 (Nec-1) is an alkaloid that blocks the activity of receptor-interacting protein 1 (RIP-1) kinase, reduces programmed necrosis in many forms of disease and ultimately plays an important role[7]. However, there are few extant studies on Nec-1 and PGC1-α regarding sepsis cases. Therefore, this study aims to explore the effect and mechanism of Nec-1 on mitochondrial injury in the renal tubular epithelial cells of septic mice by upregulating PGC1-α transcription.

Methods

Subjects

A total of 18 C57BL/6 mice were purchased from Beijing Luyuan Bode Biotechnology Co., LTD. (Batch number: SCXK (Beijing) -2018-0001). The mouse HK-2 renal tubular epithelial cells were purchased from Shenzhen Haodi Huatuo Biotechnology Co., LTD. All mice were placed in an SPF animal house with a room temperature of 24±2 °C. They could eat and drink freely before being placed in a metabolic cage 24 hours before the experiment.

Main reagents and instruments

A BCA protein quantitative kit was purchased from Beijing DingGuo Changsheng Biotechnology Co., LTD. In addition, the following instruments and reagents were used. An automatic biochemical analyser (Nanjing Xinhuitong Biotechnology Co., LTD.), lipopolysaccharides (LPS) (Xiamen Lunchangshuo Biotechnology Co., LTD.), mouse anti-PGC1-α monoclonal antibody (Shanghai Yuanmu Biotechnology Co., LTD.), PCR primers (Yunnan YiTong Experimental Education Technology Co., LTD.), a microplate reader (Molecular Devices), PVDF membranes (Wuhan Khayal Biotechnology Co., LTD.), and Tizol reagent (Qingdao Jisskang Biotechnology Co., LTD). Other instruments used were an automatic biochemical analyser (Skillsmodel Biotechnology (Beijing) Co., LTD.), a real-time fluorescence quantitative PCR instrument (Hangzhou Bio-Genertechnology Co., LTD.), an ultra-clean workbench (Hangzhou Noding Scientific Equipment Co., LTD.), a horizontal shaker (Wuxi NEST Biotechnology Co., LTD.), a –80 °C ultra-low temperature refrigerator (Haier Biomedical and Shanghai Shiwei Experimental Instrument Technology Co., LTD.), a UV spectrophotometer (Dongguan PuBiao Experimental Equipment Technology Co., LTD.), an inverted microscope (Guangzhou Mingmei Photoelectric Technology Co., LTD.), and a fluorescent microscope (Guangzhou Koster Scientific Instrument Co., LTD.).

Methodology

• A total of 18 C57BL/6 mice were randomly and equally divided into a normal control group, an LPS group and an LPS+Nec-1 group. Mice in the normal control group were intraperitoneally injected with 10 mL·kg-1 sterile water, the LPS group were intraperitoneally injected with 10 mL·kg-1 LPS, and the LPS+Nec-1 group were intraperitoneally injected with 1.65 mL· kg-1 Nec, followed by 10 mL·kg-1 LPS. After 18 h, the mice in each group were anaesthetised using 1% pentobarbital sodium intraperitoneal injection. After a laparotomy, blood was taken from their inferior vena cava and both kidneys. After the sampling, the mice were sacrificed using the bloodletting method.

• Venous blood was taken from the mice in each group, and the upper serum was taken after centrifugation. The serum creatinine and urine nitrogen levels of the mice in each group were determined using an automatic biochemical analyser. Western blot and real-time quantitative PCR tests were conducted to determine the protein and mRNA expression levels of PGC1-α in the renal tissue of each group of mice.

• The HK-2 cells taken from the mouse renal tubular epithelial cells were randomly divided into a normal control group, an LPS group and an LPS+Nec-1 group after routine culture and passage. The normal control group cells had no treatment, LPS group cells were treated with 10 μg·mL-1 LPS for 18 h, and cells in the LPS+Nec-1 group were pre-treated with 50 μg· mL-1 Nec-1 for 6 h, followed by treatment with 10 μg· ml-1 LPS for 18 h.

• The mitochondrial morphological changes of the HK-2 cells in each group were observed by electron microscopy.

Statistical analysis

All data were analysed using the SPSS Statistics

21.0 software package, and the measurement data of serum creatinine and urine nitrogen levels in each group were expressed as ($\bar{x}\pm s$). A t-test was used for comparisons between two groups and a one-way ANOVA was used for comparing multiple groups. A P-value of <0.05 was considered statistically significant.

Results

Comparison of serum creatinine and urine nitrogen levels among groups

Compared with the normal control group, the serum creatinine and urine nitrogen levels in the LPS group increased significantly (P<0.05), while the serum creatinine and urine nitrogen levels in the LPS+Nec-1 group decreased significantly compared with the LPS group (P<0.05) (see Table 1).

Group	Serum Creatinine (μmol/L)	Urine Nitrogen (mmol/L)
Normal control group	9.+91±1.95	13.98±4.94
LPS group	30.19±4.29[a]	32.65±5.57[a]
LPS+Nec-1 group	11.61±1.78[b]	14.26±4.25[b]

Table 1: Comparison of serum creatinine and urine nitrogen levels in each group ($\bar{x}\pm s$).
Note: [a]was compared with the normal control group (P<0.05), [b]was compared with the LPS group (P<0.05).

Comparison of morphological changes in the renal tubular epithelial cells of each group

The epithelial structure of the renal tubules in the normal control mice did not change significantly. However, the number of mitochondria in the renal tubule epithelial cells of mice in the LPS group decreased significantly. In addition, no mitochondrial crests were observed and there was also mitochondrial swelling and vacuolation. The degree of mitochondrial swelling in the renal tubular epithelial cells in the LPS+Nec-1 group was minor by contrast; the number of mitochondria did not change significantly and no mitochondrial vacuolation or mitochondrial crest disappearance was observed.

Comparison of PGC1-α protein and mRNA expression levels in the renal tissues of each group

Compared with the normal control group, the protein and mRNA expression levels of PGC1-α in kidney tissue of mice in the LPS group decreased significantly (P<0.05). By contrast, the protein and mRNA expression levels of PGC1-α in the renal tissues of mice in the LPS+Nec-1 group increased significantly (P<0.05) (see Table 2).

Group	Protein Expression Levels	mRNA Expression Levels
Normal control group	1.01±0.39	1.02±0.24
LPS group	0.68±0.15[a]	0.42±0.11[a]
LPS+Nec-1 group	1.15±0.25[b]	0.82±0.23[b]

Table 2: Comparison of PGC1-α protein and mRNA expression levels in the renal tissues of each group ($\bar{x}\pm s$).
Note: [a]was compared with the normal control group (P<0.05), [b]was compared with the LPS group (P<0.05).

Discussion

Studies on septic acute kidney injuries in animals and on the renal tissue of patients with septic acute kidney injury showed that renal tubular epithelial cell necrosis was rare. Therefore, we speculated that renal tubular epithelial cell necrosis could not be assumed to be the main cause of septic acute kidney injury[8]. Previous studies on septic acute kidney injury mainly focused on mitochondrial damage and microcirculation disorders, but the relevant mechanisms were not fully understood. Therefore, this study aimed to explore the protective effect and mechanism of Nec-1 on mitochondrial injury in renal tubular epithelial cells. Nec-1 is a RIP-1 kinase inhibitor that can block the phosphorylation of RIP-1 to RIP-3 and ultimately prevent programmed necrosis[9]. Studies of ischaemia-reperfusion acute kidney injury have reported that Nec-1 can block RIP-1 kinase activity, which can reduce the levels of programmed necrosis in renal tubular epithelial cells and ultimately alleviate the degree of kidney injury[10]. Other studies have shown that Nec-1 inhibits RIP-1 and activates RIP-3, leading to the inhibition of mitochondrial division and permeability conversion. PGC1-α, as a co-stimulator, was assumed to 'control' the mitochondria[11].

Studies related to acute kidney injury have shown that a reduced expression level of PGC1-α in this disease can lead to mitochondrial functional structure destruction and regeneration disorders[12]. Other reports have shown that when septic acute kidney injury occurs, the expression levels of PGC1-α can be reduced, which can lead to the destruction of the mitochondrial functional structure in renal tubular epithelial cells, further aggravating the degree of kidney injury[13]. Animal experiments showed that when sepsis complicates an acute kidney injury, the level of PGC1-α in mouse renal tubular epithelial cells decreases, while the prognosis for the kidney itself also worsens. Some mice with a sustained level of PGC1-α mostly

developed persistent mitochondrial destruction and irreversible renal failure[14]. Other studies have shown that deleting the PGC1-α gene in mice can further increase the degree of mitochondrial damage and deterioration of renal function in renal tubular epithelial cells during sepsis. Upregulating the expression of PGC1-α expression in mice can significantly improve the mitochondrial function of renal tubular epithelial cells, increase the number of mitochondria, and ultimately achieve the goal of kidney protection[15].

In this study, we found that Nec-1 significantly improved the levels of serum creatinine and urinary nitrogen in LPS-induced septic acute kidney injury mice, so we speculated that Nec-1 may have a protective effect on the kidneys during instances of septic acute kidney injury mice. We also found that the number of mitochondria in renal tubule epithelial cells of mice in the LPS group was significantly reduced, and there was significant mitochondrial damage including mitochondrial swelling and vacuolation. The number of mitochondria in renal tubule epithelial cells of mice in the LPS+Nec-1 group increased significantly, and the degree of mitochondrial damage also significantly improved. These results suggest that mitochondrial damage may be involved in the pathogenesis of septic acute kidney injury and that Nec-1 can significantly alleviate the degree of mitochondrial damage in cases of septic acute kidney injury in mice. The results also showed that the expression levels of PGC1-α protein and mRNA in the renal tissues of mice in the LPS+Nec-1 group were significantly increased ($P<0.05$), which suggests that Nec-1 can alleviate mitochondrial damage in mice by increasing the transcription level and protein expression level of PGC1-α.

In summary, Nec-1 can improve the degree of mitochondrial damage in the renal tubular epithelial cells of mice with sepsis, and also exhibits a renal protective function, possibly through upregulation of PGC1-α transcription.

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