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CIAPIN1 attenuates ferroptosis via regulating PI3K/AKT pathway in LPS-induced podocytes

Ziqing Zhang[1†], Jinmiao Ma[1†], Minyu Shi[1], Jingcong Huang[1] and Zhenyu Xu[1*]

Abstract

Objective Cytokine-induced apoptosis inhibitor 1 (CIAPIN1) is a crucial anti-apoptotic protein; however, its role and associated molecular pathways in ferroptosis remain largely unexplored. This study aimed to investigate the effects of CIAPIN1 on ferroptosis in lipopolysaccharide (LPS)-induced podocytes and the associated underlying phenomenon.

Methods In this study, we recruited 50 sepsis patients (aged 56.63 ± 10.33) with acute kidney injury (AKI), 50 sepsis patients without AKI, and 50 healthy controls. We established an in vitro model of LPS-induced MPC5 podocytes. RT-qPCR and Western blotting were used to evaluate mRNA and protein expression, respectively.

Results Serum CIAPIN1 is downregulated in patients with septic AKI and LPS-induced podocytes. CIAPIN1 overexpression (OE-CIAPIN1) attenuated cell proliferation and apoptosis in LPS-induced podocytes. OE-CIAPIN1 elevated phosphorylated phosphoinositide 3-kinase (p-PI3K; p85, Tyr458) and phosphorylated protein kinase B (p-Akt; Ser473) levels in LPS-induced podocytes. OE-CIAPIN1 significantly elevated synaptopodin mRNA levels and remarkably lowered desmin mRNA expression in MPC5 cells. In contrast, treatment with the PI3K/Akt pathway inhibitor, LY294002, reversed synaptopodin and desmin mRNA expression in MPC5 cells. Additionally, OE-CIAPIN1 reduced the malondialdehyde (MDA) content and Fe2 + concentration in the lysate of MPC5 cells, while elevating the MDA content and Fe2 + concentration by LY294002 treatment. Furthermore, OE-CIAPIN1 increased ferroptosis-related proteins, including solute carrier family 7 member 11 (SLC7A11) and glutathione peroxidase 4 (GPX4), in MPC5 cells, which was reversed by LY294002 treatment.

Conclusion These results suggest that serum CIAPIN1 inhibits LPS-induced ferroptosis in podocytes by regulating the PI3K/AKT signaling pathway.

Keywords CIAPIN1, Ferroptosis, LPS, Acute kidney injury, Podocytes, PI3K/AKT

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Introduction

Ferroptosis is a programmed cell death characterized by iron-dependent lipid peroxidation and excessive accumulation of reactive oxygen species (ROS). This process distinguishes itself from other forms of cell death such as apoptosis, necrosis, and pyroptosis [1, 2]. When exposed to the ferroptosis inducer elastin, cells undergo specific morphological changes, including cell membrane rupture, reduced mitochondrial volume, increased membrane density, and loss of mitochondrial cristae [3]. Glutathione peroxidase 4 (GPX4) regulates lipid peroxidation and influences cells' sensitivity to ferroptosis [4]. Solute carrier family 7 member 11 (SLC7A11) and GPX4 are among the most important targets in ferroptosis [5]. This form of cell death is involved in a variety of pathological and physiological events, and its potential application in cancer therapy is currently under active investigation [6–8]. Numerous studies have also established links between ferroptosis and various cardiovascular disorders, including cardiomyopathy, myocardial infarction, ischemia/reperfusion injury, and heart failure [9–12].

The phosphatidylinositol-3 kinase (PI3K)/ protein kinase B (AKT) signaling pathway is a crucial signaling cascade that regulates many cellular processes including cell proliferation, growth, metabolism, and motility [13]. This pathway is one of the most frequently activated pathways in cancer development and plays a crucial role in maintaining the biological characteristics of malignant cells [14]. The PI3K/AKT signaling pathway is essential for cellular proliferation during multiple myeloma (MM) progression [15]. Previous studies have suggested that inhibiting specific signaling pathways may enhance the effectiveness of cancer treatment by inducing ferroptosis [16]. The dual PI3K/ histone deacetylase (HDAC) inhibitor BEBT-908 has been shown to promote immunogenic ferroptosis in cancer cells, highlighting the complex and bidirectional interactions between the PI3K/AKT pathway and ferroptosis [17]. However, the impact of cytokine-induced apoptosis inhibitor 1 (CIAPIN1) on ferroptosis in lipopolysaccharide (LPS)-induced podocytes via the PI3K/AKT signaling pathway has not been explored.

CIAPIN1 is a recently identified anti-apoptotic factor widely expressed in various tissues and localized in both the nucleus and cytoplasm [18]. Notably, CIAPIN1 does not share significant sequence homology with well-known apoptosis regulatory proteins such as B-cell Lymphoma 2 (BCL2), various caspases, or member of the inhibitor of apoptosis (IAP) family, nor does it resemble any signal transduction molecules [19]. The primary function of CIAPIN1 is to prevent apoptosis due to the lack of cytokines [20]. When cytokines bind to their specific receptors, they activate receptor tyrosine kinases (RTKs), propagating mitogenic and anti-apoptotic signals via downstream signaling pathways. Several cytokines, including interleukin 3 (IL-3), erythropoietin, stem cell factor (CSF), and thrombopoietin, increase the expression of CIAPIN1 through the receptor tyrosine kinase (RTK)-Ras signaling pathway and transcriptional regulation. This indicates that CIAPIN1 expression depends on the activation and signaling derived from cytokines or growth factors, particularly in the IL-3-dependent murine pro-B cell line Ba/F3 [19, 20]. Recent studies have highlighted its protective role in various cellular contexts, particularly in response to stress and injury. CIAPIN1 inhibits apoptosis induced by inflammatory cytokines, making it a potential therapeutic target for diseases characterized by excessive cell death, including acute kidney injury (AKI) [21]. The pathophysiology of AKI involves diverse cellular mechanisms that lead to kidney damage and disturb the balance of fluids, electrolytes, and waste products [22]. Moreover, podocytes are significantly affected during AKI episodes and are implicated in the progression of kidney disease when they undergo apoptosis or injury [23]. However, the potential effect of CIAPIN1 on ferroptosis in LPS-induced podocytes remains unclear. Therefore, this study aimed to explore the possible effects of CIAPIN1 on ferroptosis in LPS-induced podocytes and its associated underlying phenomena.

Patients and methods

Research subjects

The study protocol was approved by the hospital's Ethics Committee. Fifty patients (aged 56.63 ± 10.33) with sepsis and acute kidney injury (AKI) were consecutively enrolled in the intensive care unit (ICU) of our hospital. Additionally, 50 patients with sepsis without AKI were included, along with serum samples from 50 healthy blood donors matched for sex and age. Sepsis was diagnosed based on the criteria established by The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3) [24]. AKI was diagnosed using the following criteria: (1) a serum creatinine increase of 1.5 times the baseline and (2) a urine output of less than 0.5 mL/kg/h for more than 6 h. The inclusion criteria for the participants were clearly defined. The exclusion criteria were as follows: (1) renal tumor or chronic kidney disease, (2) AKI caused by factors unrelated to sepsis, and (3) severe dysfunction of other critical organs.

Cell culture and treatment

Conditionally immortalized mouse podocytes (MPC5) were obtained from the American Tissue Culture Collection. MPC5 podocytes were cultured in DMEM with 10% FBS supplemented with 100 μg/mL streptomycin and 100 U/mL penicillin in a 5% CO_2 incubator at 37 ℃. Three

grouping methods were used for the MPC5 cell experiments. Method 1: MPC5 mouse podocytes were treated with 0, 5, 10, 20, 50, and 100 μg/mL LPS for 24 h. Method 2: MPC5 cells were divided into (1) control group: conventional culture, (2) LPS group (LPS: 50 μg/mL) for 24 h, (3) LPS + Vector group: cells were transfected with empty vector of lentiviral vector pCDH-CMV for 48 h, and stimulated with 50 μg/mL LPS for 24 h, and (4) LPS + OE-CIAPIN1 group: cells were transfected with pCDH-CMV vector overexpressing CIAPIN1 for 48 h, and stimulated with 50 μg/mL LPS for 24 h. Method 3: MPC5 cells were divided into (1) control group: (2) LPS + Vector group; (3) LPS + OE-CIAPIN1 group; (4) LPS + OE-CIAPIN1 + LY group: cells were transfected with pCDH-CMV vector overexpressing CIAPIN1 for 48 h, and then incubated with a PI3K/Akt pathway inhibitor LY294002 (10 μM) and 50 μg/mL LPS for 24 h.

Plasmid construct and transfection

Human CIAPIN1-cDNA sequences were cloned into the lentiviral vector pCDH-CMV (LM-8070, LMAI Bio) and packaged in 293T cells. The vector overexpressing CIAPIN1 and the empty vector were transfected into MPC5 cells using Lipo2000, according to the manufacturer's instructions. Transfection efficiency was tested using RT-qPCR and Western blotting 48 h after transfection.

CCK-8 assay

MPC5 podocytes (1×10^4 cells per well) were seeded into 96-well plates and treated with saline and various concentrations of LPS (5, 10, 20, 50, and 100 μg/mL). Additionally, MPC5 podocytes were treated with saline, LPS, LPS + Vector, and LPS + OE-CINPIN for 24 h. After treatment, 10 μL of CCK-8 solution (Beyotime, Shanghai, China) was added to each well. Following a 3-hour incubation, a microplate reader was used to measure the cell absorbance at 450 nm.

Apoptosis assay

Following the methods of a previous study, cell apoptosis was identified using the Annexin V-FITC Apoptosis Detection Kit (Beijing Solarbio Science & Technology Co., Ltd., China) [25]. The cells were detached using trypsin-EDTA and collected via centrifugation. After washing with phosphate-buffered saline (PBS), the cells were suspended in a binding buffer and centrifuged at 300 × g for 10 min. After removing the supernatant, the cells were resuspended in binding buffer at a concentration 1×10^6 cells/mL. Subsequently, 5 μL of propidium iodide (PI) was added to the cells and incubated for 5 min under the same conditions. Apoptosis was assessed using a flow cytometer (Beckman Coulter, Inc., Brea, CA, USA).

Immunofluorescence

Immunofluorescence was performed as previously described [26]. Following three washes with PBS, Mito FerroGreen (HY-D2295; MedChemExpress) and dihydroethidium (DHE) (S0063, Beyotime, Shanghai, China) were introduced. In brief, the cellular specimens were exposed to a DHE dye solution (diluted 1:300 in 1× PBS saline) for 5 min, followed by three thorough washes with cold 1× PBS. Images were captured using an inverted microscope (IX51; Olympus, Japan). Subsequently, the intensity of DHE fluorescence, which manifested as red staining, was measured using Image Pro Plus software (Media Cybernetics Inc., Bethesda, MD, USA). The cellular specimens were then subjected to DAPI staining and examined using an inverted microscope (IX51, Olympus, Japan). Mito FerroGreen and DHE were used in strict adherence to the manufacturer's specifications.

Measurement of oxidative stress

The podocyte cell lysate was acquired and subsequently preserved at -80 °C within a refrigeration unit. A commercially available kit (S0131S, Beyotime, Shanghai, China) was used to assess malondialdehyde (MDA) activity. We followed to a previously documented protocol to quantify the extent of oxidative stress [27].

Iron quantification

Ferrous ion (Fe2+) concentrations in the MPC5 cells were analyzed using spectrophotometry. Briefly, the iron standard solution and test samples were added to 96-well plates, followed by Fe assay buffer and ferrous chromogenic solution. The sample was dissolved in 2 M HCl followed by spectrophotometry at 560 nm [28].

RT-qPCR

TRIzol (Invitrogen, USA) was utilized to extract total RNA from MPC5 podocytes. Subsequently, reverse transcription was performed to synthesis of complementary DNA (cDNA) from the extracted total RNA. To achieve mRNA amplification, Real-Time Quantitative PCR (RT-qPCR) was conducted employing SYBR Green reagent (TaKaRa, Japan) on an ABI Prism 7700 Real-Time PCR system (Applied Biosystems, USA). The internal control gene GAPDH was used to normalize the relative gene expression using the $2^{-\Delta\Delta Ct}$ method [29]. Primers for Human CIAPIN1, Human Synaptopodin, Human Desmin, and Human GAPDH were meticulously designed using the widely recognized and dependable NCBI Primer-BLAST Tool, as specified in Table 1.

Western blotting

Protein samples were collected after cellular breakdown by using RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China). A BCA kit (Beyotime) was used to

Table 1 List of primer sequences used in this research

Genes	Forward primer (5′-3′)	Reverse primer (5′-3′)
CIAPIN1	GGAGTTTGGGATCTCCCCTG	ACCCGACAGAATGACATCGAA
Synaptopodin	AGTGAGGAAGAGGAAGTGCCATTG	GCTGACTGTGGTGACTGCTAGAG
Desmin	AGACCTTCTCTGCTCTCAACTTCC	CTCGCTGACAACCTCTCCATCC
GAPDH	TGGTGAAGGTCGGTGTGAAC	TTCCCATTCTCGGCCTTGAC

determine the protein concentration. An equal volume of protein (40 μg) was mixed with loading buffer (Beyotime) and denatured in a boiling water bath for 3 min. Electrophoresis commenced at 80 V for 30 min, followed by 1–2 h at 120 V once bromophenol blue reached the separation gel. Proteins were transferred onto membranes in an ice bath at 300 mA for 60 min. After rinsing the membranes for 1–2 min with a washing solution, they were either inactivated for 1 h at room temperature or sealed overnight at 4 °C. The membranes were treated with primary antibodies against CIAPIN1 (1:500, ab154904, rabbit polyclonal, Abcam), p-PI3K (Tyr458) (1:500, ab278545, rabbit monoclonal, Abcam), PI3K (1:500, ab302958, rabbit monoclonal, Abcam), p-Akt (Ser473) (1:500, ab81283, rabbit monoclonal, Abcam), Akt (1:500, ab8805, rabbit polyclonal, Abcam), Synaptopodin (1:500, ab259976, rabbit monoclonal, Abcam), Desmin (1:500, ab32362, rabbit monoclonal, Abcam), SLC7A11 (1:500, ab175186, rabbit monoclonal, Abcam), GPX4 (1:500, sc-166570, mouse monoclonal, Santa Cruz), and GAPDH (1:2000, ab9485, rabbit polyclonal, Abcam) while shaking at room temperature for 1 h. The membranes were washed thrice using a washing solution for 10 min each, both before and after 1 h of exposure to the secondary antibody at ambient temperature. Finally, the membranes were placed in the developing solution and observed using chemiluminescence imaging analysis equipment (Gel Doc XR, Bio-Rad).

Statistical analysis

Data from at least three experiments are expressed as the mean ± standard deviation (SD). Statistical analyses were performed using SPSS version 20.0 (SPSS, Chicago, IL, USA) or GraphPad Prism software (version 9.0). Differences between groups were assessed using one-way ANOVA followed by Tukey's post-hoc test. The incidence of septic among the different groups was analyzed using Fisher's exact test. Statistical significance was set at $p < 0.05$.

Results

Serum CIAPIN1 is down regulated in septic AKI patients and LPS-induced podocytes

To investigate the potential effects of serum CIAPIN1 on patients with septic acute kidney injury (AKI) and LPS-treated podocytes, we performed an enzyme-linked immunosorbent assay (ELISA) using serum samples from three groups: healthy controls, septic patients without AKI, and septic AKI patients. The results indicated that serum CIAPIN1 levels were significantly lower in septic AKI patients than those in healthy controls and non-AKI patients (Fig. 1A). Additionally, we treated MPC5 mouse podocytes with various concentrations of LPS (0, 5, 10, 20, 50, and 100 μg/mL) and incubated them for 24 h. The viability of podocytes was assessed using the CCK-8 assay. Our findings revealed that higher doses of LPS decreased the cell proliferation rate (Fig. 1B). We also conducted RT-qPCR to measure CIAPIN1 mRNA expression in LPS-induced podocytes. The data showed that higher concentrations of LPS decreased CIAPIN1 mRNA expression in these cells (Fig. 1C). Furthermore, we performed a western blot analysis to evaluate CIAPIN1 protein expression in LPS-treated podocytes, which confirmed that increasing LPS doses led to a reduction in CIAPIN1 protein expression (Fig. 1D and Fig. S1). Quantification of the western blot results supported these findings, showing consistent trends in CIAPIN1 protein expression levels in LPS-induced podocytes (Fig. 1E). Our results demonstrated that serum CIAPIN1 is downregulated in septic AKI patients and LPS-treated podocytes.

CIAPIN1 overexpression inhibited proliferation and apoptosis in LPS-induced podocytes

To evaluate the impact of CIAPIN1 overexpression on proliferation and apoptosis of LPS-induced podocytes, we transfected MPC5 cells with pCDH-CMV-CIAPIN1 or an empty vector, and RT-qPCR and Western blotting were performed 48 h after transfection. MPC5 cells were transfected with pCDH-CMV-CIAPIN1 or an empty vector and treated with LPS (50 μg/mL) for 24 h. The results showed that overexpression of CIAPIN1 (OE-CIAPIN1) increased CIAPIN1 mRNA and protein expression (Fig. 2A, B and Fig. S2). The effect of CIAPIN1 overexpression on cell viability was assessed by the CCK-8 assay, and it was found that OE-CIAPIN1 elevated the cell proliferation rate (Fig. 2C). Apoptosis was evaluated by Annexin V-FITC double staining and analyzed by flow cytometry. We observed that OE-CIAPIN1 reduced the apoptosis rate (Fig. 2D). Quantification of the apoptotic rate included early, apoptotic cells (lower right quadrant) and late apoptotic cells (upper right quadrant) which consistently lowered apoptotic cells (Fig. 2E). These results indicated that OE-CIAPIN1 attenuated the proliferation and apoptosis of LPS-treated podocytes.

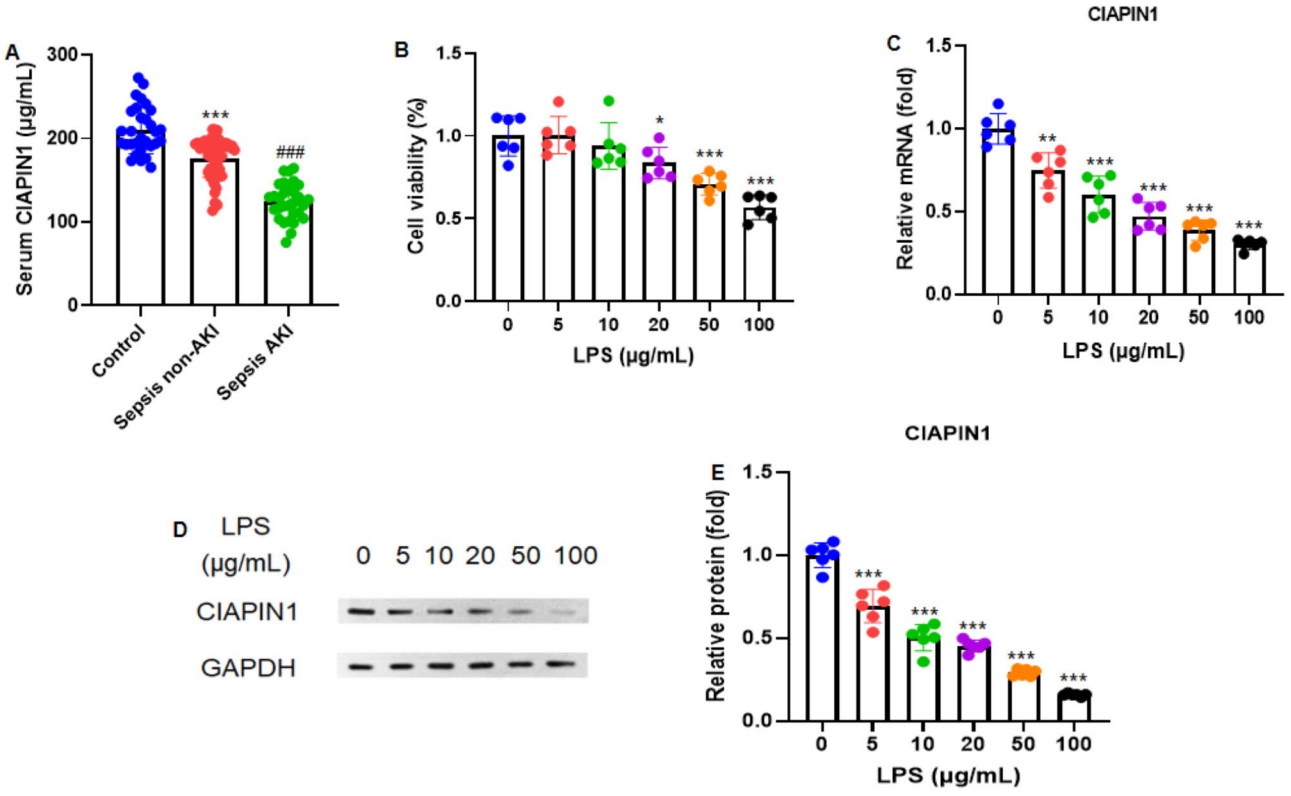

Fig. 1 Serum cytokine-induced apoptosis inhibitor 1 (CIAPIN1) is downregulated in septic acute kidney injury (AKI) patients and lipopolysaccharide (LPS)-treated podocytes. (**A**) Serum CIAPIN1 level in healthy subjects, septic patients without AKI, and septic patients with AKI. The serum samples of all subjects were measured by enzyme-linked immunosorbent assay (ELISA) ($n = 50$ in each group). (**B**) MPC5 mouse podocytes were treated with 0, 5, 10, 20, 50 and 100 μg/mL LPS for 24 h. The cell viability of podocytes were determined by CCK-8 assay. (**C**) The CIAPIN1 mRNA expression was determined by RT-qPCR in podocytes treated with LPS. (**D**) Representative gel blots of CIAPIN1 by Western blotting in podocytes treated with LPS. (**E**) These protein blots were quantified by normalizing to GAPDH. Data are presented as mean ± SD in triplicates, and analyzed using one-way ANOVA. ***$P < 0.001$ vs. control group or cell group without LPS. ###$P < 0.001$ vs. septic patients without AKI

CIAPIN1 overexpression activated PI3K/ AKT pathway in LPS-treated podocytes

In this study, we investigated the potential effects of OE-CIAPIN1 on the PI3K/ AKT pathway in LPS-induced podocytes. We performed western blot analysis to assess protein expression levels associated with the PI3K/ AKT pathway. The results indicated that OE-CIAPIN1 increased the levels of phosphorylated PI3K (p-PI3K, p85, Tyr458) and phosphorylated AKT (p-Akt, Ser473) in LPS-induced podocytes (Fig. 3A and Fig. S3). After quantifying p-PI3K (normalized to total PI3K) and p-AKT (normalized to total AKT), we observed consistent trends in the expression of both p-PI3K and p- AKT proteins (Fig. 3B, C). These findings demonstrated that OE-CIAPIN1 activates the PI3K/ AKT pathway in LPS-induced podocytes.

CIAPIN1 overexpression reduced LPS-induced podocyte injury

To examine the potential impact of OE-CIAPIN1 on LPS-induced podocyte injury, we transfected MPC5 cells with a pCDH-CMV vector overexpressing CIAPIN1 for 48 h and incubated them with the PI3K/Akt pathway inhibitor LY294002 (10 μM) and 50 μg/mL LPS for 24 h. RT-qPCR analysis was performed to measure mRNA levels of Synaptopodin and Desmin in MPC5 cells. The results showed that OE- CIAPIN1 significantly elevated synaptopodin mRNA levels while remarkably lowering desmin mRNA expression in MPC5 cells. In contrast, treatment with the PI3K/Akt pathway inhibitor, LY294002, reversed synaptopodin and desmin mRNA expression in MPC5 cells (Fig. 4A, B). In addition, we performed western blot analysis to determine the levels of Synaptopodin and Desmin proteins in MPC5 cells. OE- CIAPIN1 markedly reduced synaptopodin protein expression levels while increasing the desmin protein levels in MPC5 cells (normalized to GAPDH). LY294002 treatment altered the protein levels of synaptopodin and desmin in MPC5 cells (Fig. 4C and Fig. S4). After quantifying the synaptopodin and desmin protein bands, we found the same consistent protein expression patterns for synaptopodin and desmin in MPC5 cells (Fig. 4D, E). These results indicated that OE- CIAPIN1 attenuated LPS-induced podocyte injury in MPC5 cells.

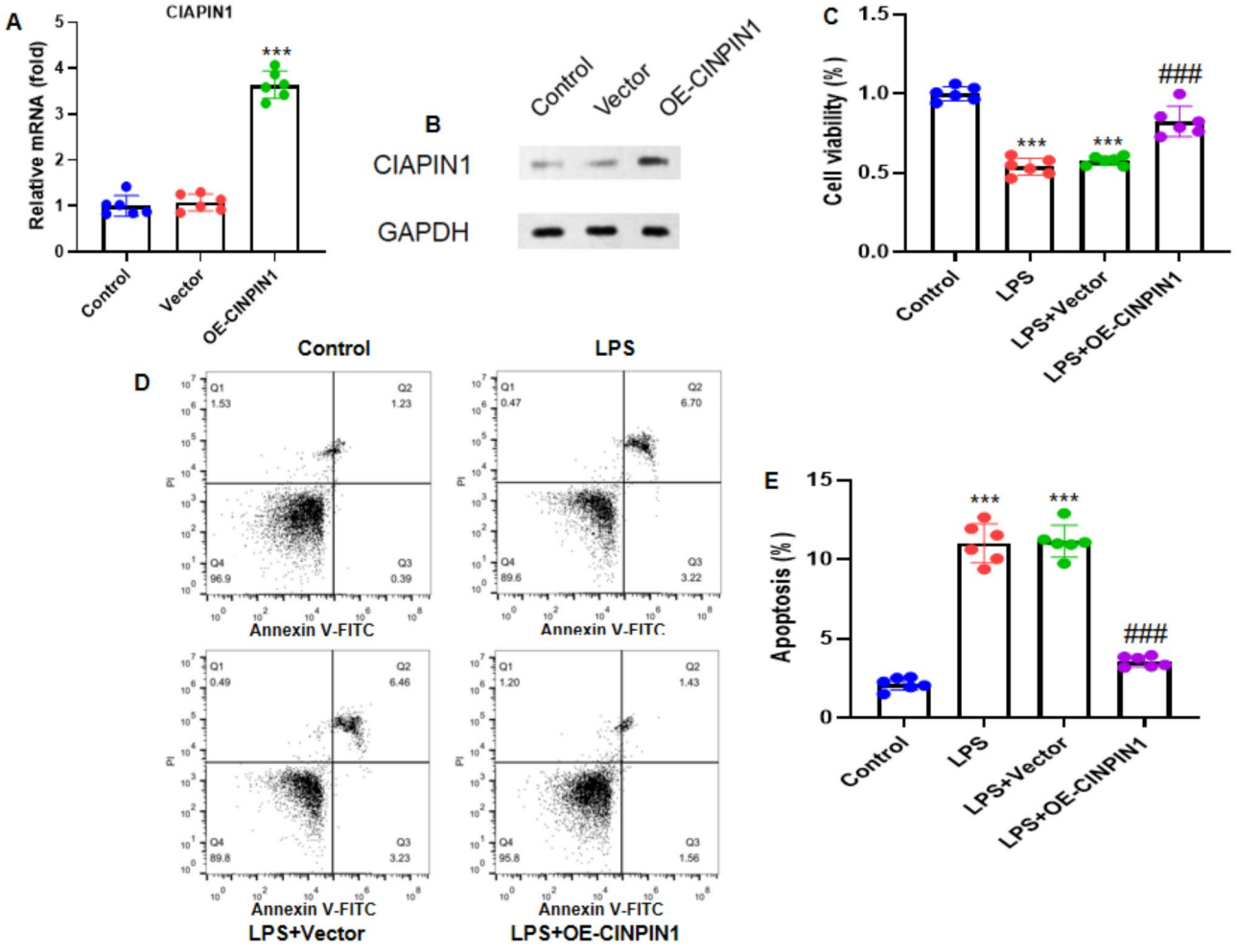

Fig. 2 CIAPIN1 overexpression attenuated proliferation and apoptosis of LPS-treated podocytes. (**A**, **B**) MPC5 cells were transfection with pCDH-CMV-CIAPIN1 or empty vector, and RT-qPCR and Western blot were performed at 48 h after transfection. MPC5 cells transfected with pCDH-CMV-CIAPIN1 or empty vector, and were treated with LPS (50 μg/mL) for 24 h. (**C**) The effect of CIAPIN1 overexpression on cell viability was assessed by CCK-8 assay. (**D**) Cell apoptosis was assessed by Annexin V-FITC double staining and analyzed by flow cytometry, and the representative images of flow cytometry are shown. (**E**) Quantification of apoptotic rate (include early apoptotic cells (lower right quadrant) and late apoptotic cells (upper right quadrant). Data are presented as mean ± SD in triplicates, and analyzed using one-way ANOVA. ***$P < 0.001$ vs. control group; ###$P < 0.001$ vs. LPS + Vector group

CIAPIN1 overexpression suppressed cellular ROS production and ferroptosis in LPS-induced podocytes

In this study, we explored the possible effect of OE-CIAPIN1 on cellular ROS production and ferroptosis in LPS-induced podocytes. We stained MPC5 cells with DHE and observed that the LPS + vector increased the proportion of DHE-positive cells compared to the control, which was decreased by LPS + OE-CIAPIN1 treatment. Additionally, LPS + OE-CIAPIN1 + LY294002 treatment elevated the number of DHE-positive cells (Fig. 5A). After quantifying DHE-positive cells relative to DAPI-positive cells, we observed the same trend (Fig. 5B). In addition, the colorimetric method was used to determine the MDA content, an oxidative stress indicator MDA content and the concentration of Fe2 + was measured in the lysate of MPC5 cells. The results showed that OE-CIAPIN1 reduced the MDA content and Fe2 + concentration in the lysate of MPC5 cells, while increasing the MDA content and Fe2 + concentration by LY294002 treatment (Fig. 5C, D). Furthermore, western blot analysis was performed to determine the levels of ferroptosis-related proteins, such as SLC7A11 and GPX4, in MPC5 cells. The data indicated that OE-CIAPIN1 increased SLC7A11 and GPX4 protein expression in MPC5 cells (Normalized to GAPDH), which was reversed by LY294002 treatment (Fig. 5E and Fig. S5). After quantifying SLC7A11 and GPX4 protein bands, we observed consistent expression patterns of SLC7A11 and GPX4 in MPC5 cells (Fig. 5F, G). These results demonstrated that OE-CIAPIN1 inhibited cellular ROS production and ferroptosis in LPS-induced podocytes.

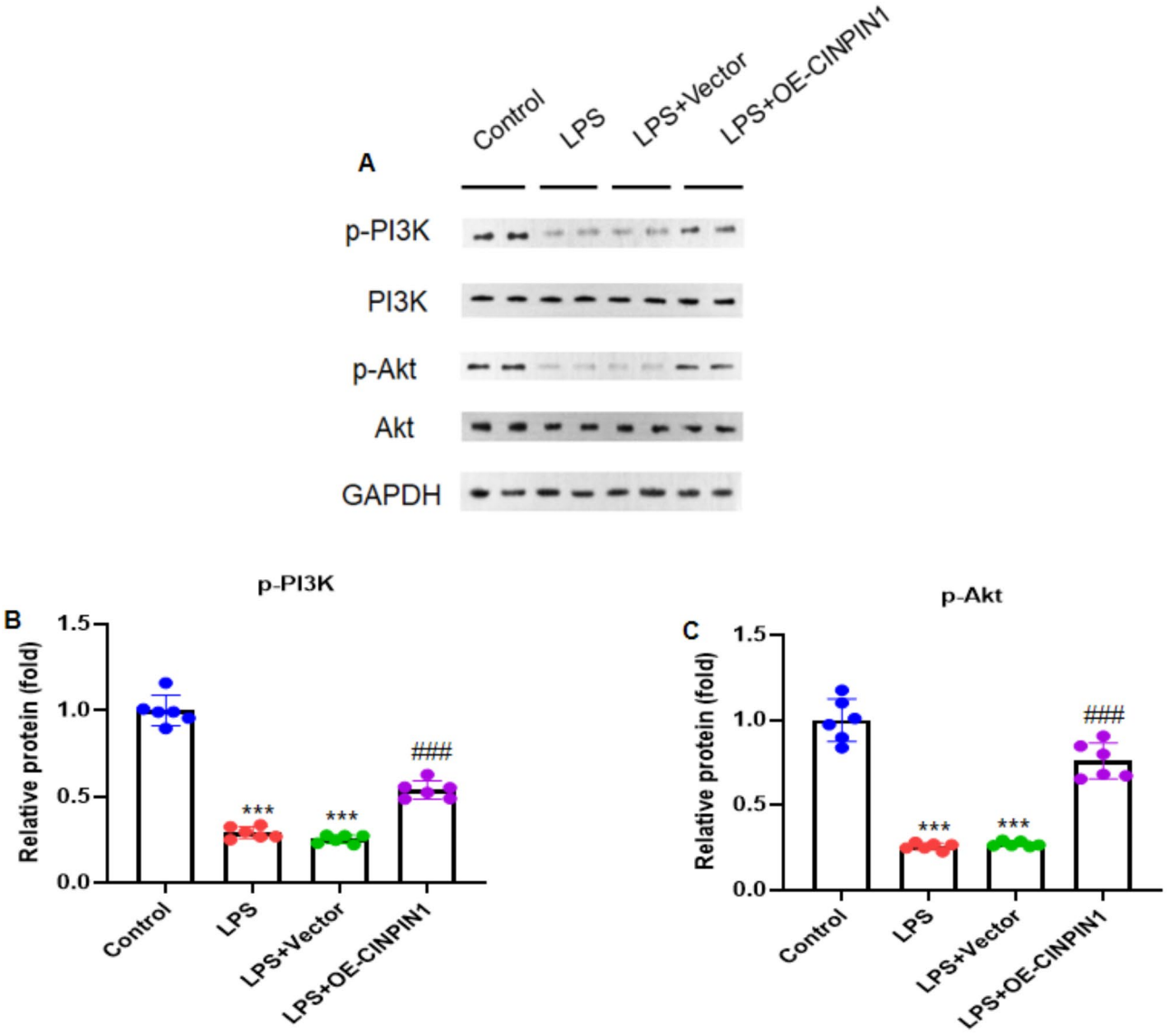

Fig. 3 CIAPIN1 overexpression activates phosphoinositide 3-kinase (PI3K)/ protein kinase B (Akt) pathway in LPS-treated podocytes. (**A**) Representative gel blots depicting levels of phosphorylated PI3K (p-PI3K, p85, Tyr458) and phosphorylated Akt (p-Akt, Ser473). (**B**, **C**) Quantification analysis of p-PI3K (normalized to total PI3K) and p-Akt (normalized to total Akt). Data represent the average of three independent experiments (mean ± SD). ***$P < 0.001$ vs. control group; ###$P < 0.001$ vs. LPS + Vector group

Discussion

This study examined the potential effects of CIAPIN1 on ferroptosis in podocytes induced by LPS and the associated molecular phenomena. Our findings indicate that serum levels of CIAPIN1 were reduced in patients with septic AKI and LPS-induced podocytes. Furthermore, OE-CIAPIN1 inhibited cell proliferation and apoptosis, activated the PI3K/AKT signaling pathway, and reduced LPS-induced podocyte injury. Additionally, OE-CIAPIN1 decreased cellular reactive oxygen species (ROS) production and reduced ferroptosis in LPS-induced podocytes. These results suggest that serum CIAPIN1 levels may influence ferroptosis through the PI3K/AKT signaling pathway in LPS-induced podocytes.

Ferroptosis is a recently identified modality of cellular demise associated with many apoptotic pathways and is characterized by lipid peroxidation and iron accumulation. Ferroptosis involves the intricate regulation of many genes and signaling cascades, culminating in complex biochemical reactions [30, 31]. Contemporary investigations have elucidated the effectiveness of ferroptosis inducers in eradicating neoplastic cells [32]. In contrast to their regular counterparts, cancerous cells demonstrate heightened iron dependencies, and this reliance on iron amplifies their vulnerability to ferroptosis

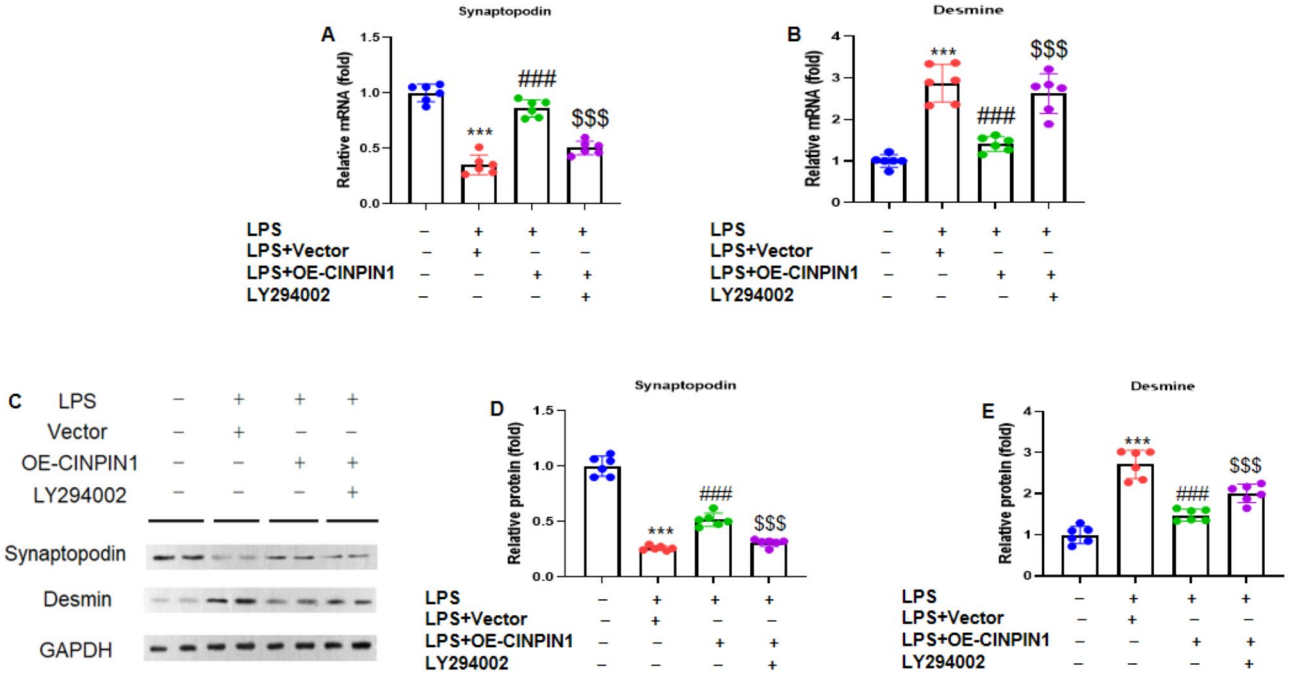

Fig. 4 CIAPIN1 overexpression attenuates LPS-induced podocyte injury. MPC5 cells were transfected with pCDH-CMV vector overexpressing CIAPIN1 for 48 h, and then incubated with a PI3K/Akt pathway inhibitor LY294002 (10 μM) and 50 μg/mL LPS for 24 h. (**A**, **B**) RT-qPCR was performed to measure the mRNA levels of Synaptopodin and Desmin in MPC5 cells. (**C**) Representative gel blots depicting levels of Synaptopodin and Desmin proteins in MPC5 cells (Normalized to GAPDH). (**D**, **E**) Quantification analysis of Synaptopodin and Desmin protein bands. Data represent the average of three independent experiments (mean ± SD). ***$P < 0.001$ vs. control group; ###$P < 0.001$ vs. LPS + Vector group; $$$$P < 0.001$ vs. LPS + OE- CIAPIN1 group

[33]. In this study, we established a connection between CIAPIN1 and ferroptosis in podocytes, highlighting the importance of ferroptosis in podocyte injury. Ferroptosis is a form of nonapoptotic cell death triggered by ROS accumulation [34, 35]. In the context of morphological changes, ferroptosis leads to a reduction in mitochondrial volume, an increase in mitochondrial membrane density, and the disintegration of mitochondrial cristae within cellular structures [36]. The proteins GPX4 and SLC7A11 are recognized as critical regulators of ferroptosis; reduced levels of GPX4 and SLC7A11 result in the accumulation of ROS, promoting ferroptosis process [37–39]. A decrease in GPX4 and SLC7A11 expression disrupts the balance between ROS production and elimination [36]. However, our results showed that OE-CIAPIN1 reduced ROS, MDA, and Fe2 + levels in LPS-induced podocytes. Moreover, OE-CIAPIN1 effectively increased SLC7A11 and GPX4 expression levels in LPS-induced podocytes. However, LY294002 treatment effectively reversed these expression patterns. These results suggested that OE-CIAPIN1 inhibits cellular ROS production and ferroptosis in LPS-induced podocytes.

We identified the PI3K/AKT signaling pathway as a critical anti-inflammatory phenomenon in our experimental investigations. AKT is a crucial downstream component of the PI3K signaling cascade and is involved in several cellular functions, including proliferation, differentiation, and anti-apoptotic processes [39–41]. Research has shown that the phosphorylation of AKT reduces apoptosis in cardiomyocytes induced by sepsis [42]. Furthermore, inhibition of the PI3K/AKT signaling pathway has been observed to diminish the mesenchymal trans-differentiation of podocytes [43]. A previous study demonstrated that activation of the PI3K/AKT signaling pathway significantly decreased Ang II-induced podocyte apoptosis [44]. An earlier investigation indicated that LPS inhibits AKT phosphorylation, whereas fractalkine (FKN) depletion activates it. This depletion ameliorated LPS-induced acute kidney injury (AKI) by promoting the activation of the PI3K/AKT signaling pathway. In cultured podocytes, knocking down FKN inhibited podocyte apoptosis by enhancing AKT phosphorylation; this effect was reversed when a PI3K/AKT inhibitor was also applied [45]. However, our study demonstrated that LPS attenuates the phosphorylation of PI3K and AKT, whereas CIAPIN1 overexpression activates it. These results suggest that OE-CIAPIN1 activates the PI3K/AKT pathway in LPS-induced podocytes. OE-CIAPIN1 also mitigated LPS-induced podocyte injury, an effect reversed by the PI3K/AKT inhibitor (LY294002) was used in MPC5 cells. To determine the dose of the PI3K/Akt pathway inhibitor, LY294002, the concentration-dependent effects of LY294002 (5μM, 10μM, and 20μM)

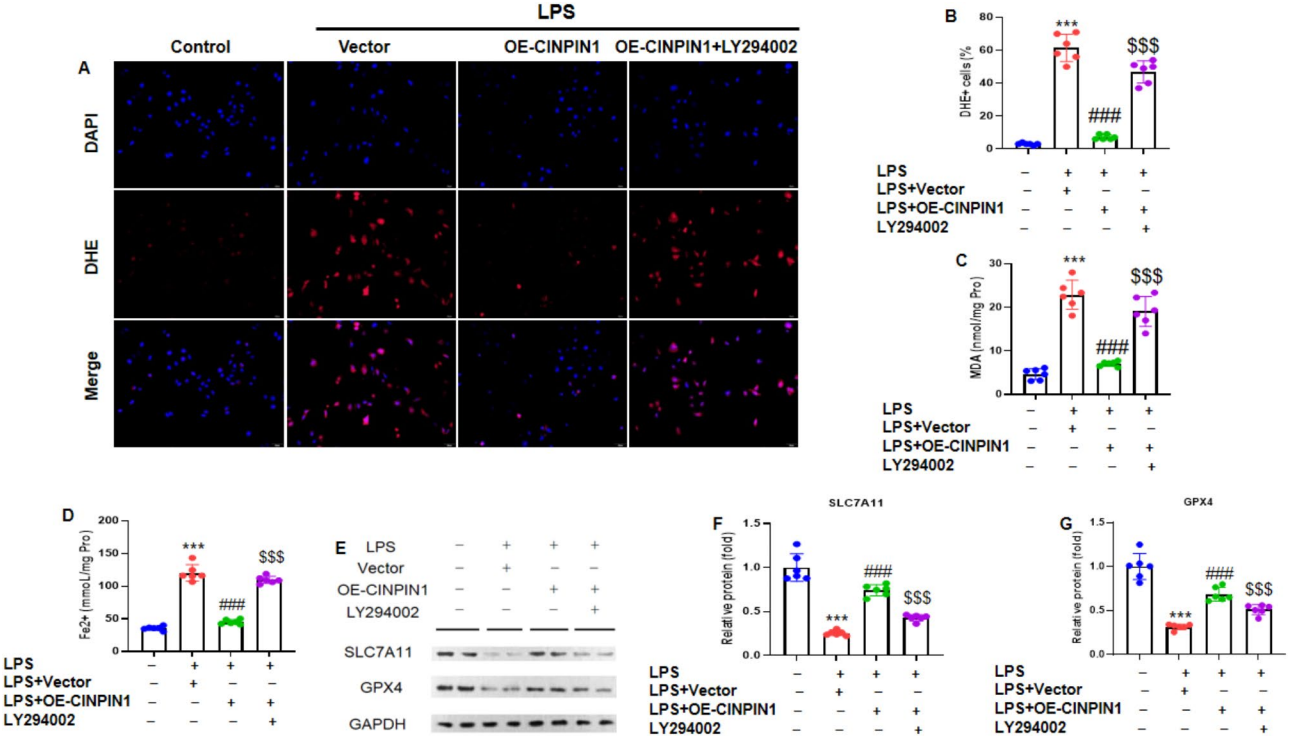

Fig. 5 CIAPIN1 overexpression suppresses cellular reactive oxygen species (ROS) production and ferroptosis in LPS-induced podocyte. (**A**) MPC5 cells were stained with DHE, and representative images are shown (magnification 100×). (**B**) Quantification of DHE positive cells relative to DAPI positive cells. (**C**) The colorimetric method was used to determine oxidative stress indicators malondialdehyde (MDA) content in lysate of MPC5 cells. (**D**) The concentration of Fe^{2+} were measured in MPC5 cells. (**E**) Representative gel blots depicting levels of ferroptosis-related proteins in MPC5 cells (Normalized to GAPDH). (**F**, **G**) Quantification analysis of solute carrier family 7 member 11 (SLC7A11) and glutathione peroxidase 4 (GPX4) protein bands. Data are presented as mean ± SD in triplicates, and analyzed using one-way ANOVA and Bonferroni test was used for the post-hoc test. ***$P < 0.001$ vs. control group; ###$P < 0.001$ vs. LPS + Vector group; $$$$P < 0.001$ vs. LPS + OE- CIAPIN1 group

were analyzed (Fig. S6). Based on the concentration-dependent effects of LY294002, we selected 10μM for this study.

CIAPIN1 is a recently identified anti-apoptotic factor that is widely expressed in various tissues and is found in both the nucleus and cytoplasm [18]. Its primary role is to prevent apoptosis due to cytokine deficiency [20]. When cytokines bind to their specific receptors, they activate RTKs, propagating mitogenic and anti-apoptotic signals through the downstream signaling pathways. Several cytokines, including IL-3, erythropoietin, stem cell factor, and thrombopoietin, increase the expression of CIAPIN1 via the RTK-Ras signaling pathway and transcriptional regulation [19, 20]. However, the present investigation showed that serum CIAPIN1 levels were lower in patients with septic AKI and in podocytes induced by LPS. Moreover, OE-CIAPIN1 inhibited cell proliferation and decreased apoptosis. It also activates the PI3K/AKT signaling pathway and alleviates LPS-induced podocyte injury. Additionally, OE-CIAPIN1 reduced ROS production and mitigated ferroptosis in LPS-induced podocytes.

Our study had several inherent limitations. (1) Caution is advised when interpreting our results because of the relatively small size of our research sample. Further research should be conducted using a large sample size to verify the study results. (2) In the current study, ferroptosis inhibitors were not used as a treatment group or activators as positive controls to confirm that LPS-induced podocyte death is ferroptosis. In future research, the results will be validated using ferroptosis inhibitors. (3) The study was not designed as a prospective longitudinal analysis; instead, it was structured as an in vitro investigation. Therefore, the prognostic significance of CIAPIN1 requires further validation.

In summary, our investigation speculated that serum CIAPIN1 protects against LPS-induced ferroptosis in vitro. Serum CIAPIN1 inhibits LPS-induced ferroptosis in podocytes by regulating the PI3K/AKT signaling pathway. This study may offer a new therapeutic approach for diseases related to ferroptosis. Future research should examine how mitochondrial dysfunction contributes to ferroptosis and its implications for various diseases.

Supplementary Information

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Supplementary Material 1

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Author contributions
Z.Z. and J.M. Conceptualization, Methodology, Data curation, Visualization, Investigation, Writing-original draft. M.S. and J.H. Methodology, Data curation, Visualization, Investigation. Z.X. Project administration, Supervision, Funding acquisition, Resources, Writing-review & editing.

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Data availability
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical approval
This study was approved (**2024-AR-025**) by Seventh People's Hospital of Shanghai University of Traditional Chinese Medicine Ethics Committee. The authors envisaged all standard protocols in accordance with the 1964 Declaration of Helsinki.

Consent to participate
Written informed consent was obtained from all subjects involved in the study.

Consent for publication
Patients was agreed to publish their specimen data.

Competing interests
The authors declare no competing interests.

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