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Moderate Hypothermia Alleviates Sepsis-Associated Acute Lung Injury by Suppressing Ferroptosis Induced by Excessive Inflammation and Oxidative Stress via the Keap1/GSK3β/Nrf2/GPX4 Signaling Pathway

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Purpose: Sepsis-associated acute lung injury (SA-ALI) is a common complication in patients with sepsis, contributing to high morbidity and mortality. Excessive inflammation and oxidative stress are crucial contributors to lung injury in sepsis. This study aims to examine the protective effects of moderate hypothermia on SA-ALI and explore the underlying mechanisms.

Methods: Sepsis was induced in rats through cecal ligation and puncture followed by intervention with moderate hypothermia (32–33.9°-C). Blood, bronchoalveolar lavage fluid, and lung tissues were collected 12 hours post-surgery. Inflammatory responses, oxidative injury, SA-ALI-related pathophysiological processes, and Keap1/GSK3β/Nrf2/GPX4 signaling in septic rats were observed by ELISA, lung W/D ratio, immunohistochemistry, immunofluorescence, histological staining, Western blotting, RT-qPCR, and TEM assays.

Results: Moderate hypothermia treatment alleviated lung injury in septic rats, reflected in amelioration of pathological changes in lung structure and improved pulmonary function. Further, moderate hypothermia reduced arterial blood lactate production and suppressed the expression of inflammatory factors IL-1β, IL-6, and TNF-α; downregulated ROS, MDA, and redox-active iron levels; and restored GSH and SOD content. TEM results demonstrated that moderate hypothermia could mitigate ferroptosis in PMVECs within lung tissue. The underlying mechanism may involve regulation of the Keap1/Nrf2/SLC7A11/GPX4 signaling pathway, with the insulin pathway PI3K/Akt/GSK3β also playing a partial role.

Conclusion: Collectively, we illustrated a novel potential therapeutic mechanism in which moderate hypothermia could alleviate ferroptosis induced by excessive inflammation and oxidative stress via the regulation of Keap1/GSK3β/Nrf2/GPX4 expression, hence ameliorating acute lung injury in sepsis.

Keywords: moderate hypothermia, sepsis, acute lung injury, ferroptosis, Nrf2, inflammation

Introduction

Sepsis is a life-threatening form of organ dysfunction caused by dysregulated response to infection.[1] Sepsis poses a serious threat to human health, with an estimated 48.9 million cases recorded worldwide, resulting in 11.0 million deaths and accounting for 19.7% of global mortality.[2] The lung is the most vulnerable target organ in sepsis. Sepsis-associated acute lung injury (SA-ALI) occurs earliest and most severely, making it one of the key factors affecting the prognosis of sepsis.[3] Upon invasion of foreign pathogens, alveolar macrophages (AMs) are activated and polarized into the pro-inflammatory M1 phenotype, which secrete significant amounts of inflammatory cytokines such as interleukin 1 (IL-1), interleukin 6 (IL-6), and tumor necrosis factor-α (TNF-α). These inflammatory cytokines further induce the production of reactive oxygen species (ROS).[4–6] Excessive inflammatory cytokines and ROS attack the cell membrane and DNA of pulmonary microvascular endothelial cells (PMVECs).[7] Impaired PMVECs increase vascular permeability, leading to the infiltration of more

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inflammatory cells (bone marrow-derived monocytes and neutrophils), which exacerbate tissue damage.[8] Therefore, the alleviation of excessive inflammation and oxidative stress as well as the maintenance of cellular function and barrier integrity are important therapeutic strategies in the treatment of SA-ALI.

Ferroptosis, an iron-dependent, regulated form of cell death, is initiated by the lethal accumulation of lipid ROS due to impairment of the glutathione (GSH)-dependent lipid peroxide repair system.[9] A clinical study reported a strong association between ferroptosis and multiorgan dysfunction syndrome (MODS), which is prevalent among patients with sepsis. The plasma levels of catalytic iron and malondialdehyde, a marker of lipid peroxidation, were found to be positively correlated with sequential organ failure assessment (SOFA) score in 176 critically ill adult patients.[10] Vascular leakage during sepsis leads to multiple organ damage, closely linked to alterations in gene expression and metabolomics indicative of ferroptosis.[11] Ferroptosis plays a significant role in sepsis-induced organ injury involving the heart, lungs, and brain. Inhibiting ferroptosis has proven effective in attenuating sepsis-associated multiple organ failure.[12–14] Furthermore, notably, nuclear factor erythroid 2-related factor 2 (Nrf2), a master regulator of the antioxidant response, can regulate various cytoprotective genes, including glutathione peroxidase 4 (GPX4).[15,16] Upregulation of Nrf2 would exert protective effects in lung injury by inducing the expression of GPX4 and alleviating ferroptosis.[13,17]

The activity of the Nrf2 protein is mainly determined by its stability.[18] Under normal physiological conditions, Nrf2 typically exhibits a short half-life of approximately 10–30 minutes. The primary negative regulator of Nrf2 is Kelch-like ECH-associated protein 1 (Keap1), which mediates the continuous ubiquitylation and subsequent proteasomal degradation of Nrf2, thereby maintaining extremely low basal levels.[19,20] In response to oxidative stress, reactive cysteine residues in Keap1 are oxidized, leading to Keap1 inactivation, increased stabilization of Nrf2, and its subsequent translocation into the nucleus, thereby activating the expression of downstream antioxidant genes such as *SLC7A11* and *GPX4*.[21–25] Independent of Keap1, glycogen synthase kinase-3β (GSK-3β) is another important negative regulator of Nrf2. GSK-3β regulates the stability of Nrf2 by phosphorylating serine residues, thereby promoting Nrf2 degradation through the β-transducing repeat-containing protein (β-TrCP)-mediated ubiquitin proteasomal system.[26]

The use of hypothermia therapy for treating hypoxic-ischemic encephalopathy is steadily increasing in clinical practice, accompanied by growing interest in its application for sepsis.[27,28] Fluctuations in body temperature can affect inflammation and immune function, with variable effects on patient outcomes.[29] An increase in core temperature can lead to neutrophil accumulation, elevated levels of inflammatory cytokines, and contribute to pulmonary vascular injury.[30] Uncontrolled fever is associated with poor prognosis in patients with sepsis, while treatments that induce hypothermia offer clinical benefits to these patients.[31,32] However, other scholars argue that hypothermia does not reduce mortality in patients with sepsis.[33] Therefore, it is essential to further investigate the potential role of hypothermia in this context and to explore the underlying and mechanisms. Our previous study indicated that hypothermia can inhibit inflammatory responses in rats via the TLR2/MyD88 axis and suppress macrophage pyroptosis through the Nrf2/ROS/NF-κB pathway, thereby attenuating septic lung injury (some results remain unpublished).[34,35] However, whether hypothermia can protect lung tissue from ferroptosis via activation of the Nrf2 factor is still unclear. To address this gap, in this study, we investigate the protective role of hypothermia in SA-ALI and the involvement of the Keap1/GSK3β/Nrf2/GPX4 signaling axis.

Material and Methods

Ethical Inspection and Experimental Animals

The animal research protocol was approved by the Animal Care and Welfare Committee of Guangxi Medical University (Protocol license number: 202306012). All animals were treated according to the guidelines established for the Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MD, USA). Sixty male Sprague-Dawley wild-type (WT) rats, aged between 8 and 12 weeks and weighing 300 to 400 g, were provided by the Laboratory Animal Centre of Guangxi Medical University.

Rat Model of Cecal Ligation and Puncture (CLP)

Multimicrobial sepsis was induced in Sprague-Dawley rats via cecal ligation and puncture (CLP) in the absence of antibiotics, as previously described by Rittirsch et al.[36] Briefly, rats were first anesthetized with 3% sodium pentobarbital

in saline (50 mg/kg, Sigma-Aldrich, Germany) via intraperitoneal injection; then, a midline incision was made using aseptic technique. The blind sac of the cecum was isolated and ligated with 4–0 suture at the cecal midpoint (ie, 50% cecal ligation). The cecum was punctured twice with an 18-gauge needle midway between the cecal ligation and the tip, in a mesenteric-to-antimesenteric direction, and gently squeezed to express one drop of fecal matter to ensure proper puncture (Figure 1A). The sham-operation group underwent a similar procedure, except without the CLP. All rats were given 5 mL/100 g body weight of saline subcutaneously after the incision was sutured, and fluid resuscitation was performed again 6 hours postoperatively.

Targeted Temperature Management (TTM)

The rectal temperature of the rats was continuously monitored using an electronic thermometer with a digital display during the temperature management period. Rats in the hypothermia group were transferred to a homeothermic pad (ALC-HTP Homeothermic System, Alcott Biotech Co., Ltd., Shanghai, China) after the operation and maintained at the target temperature

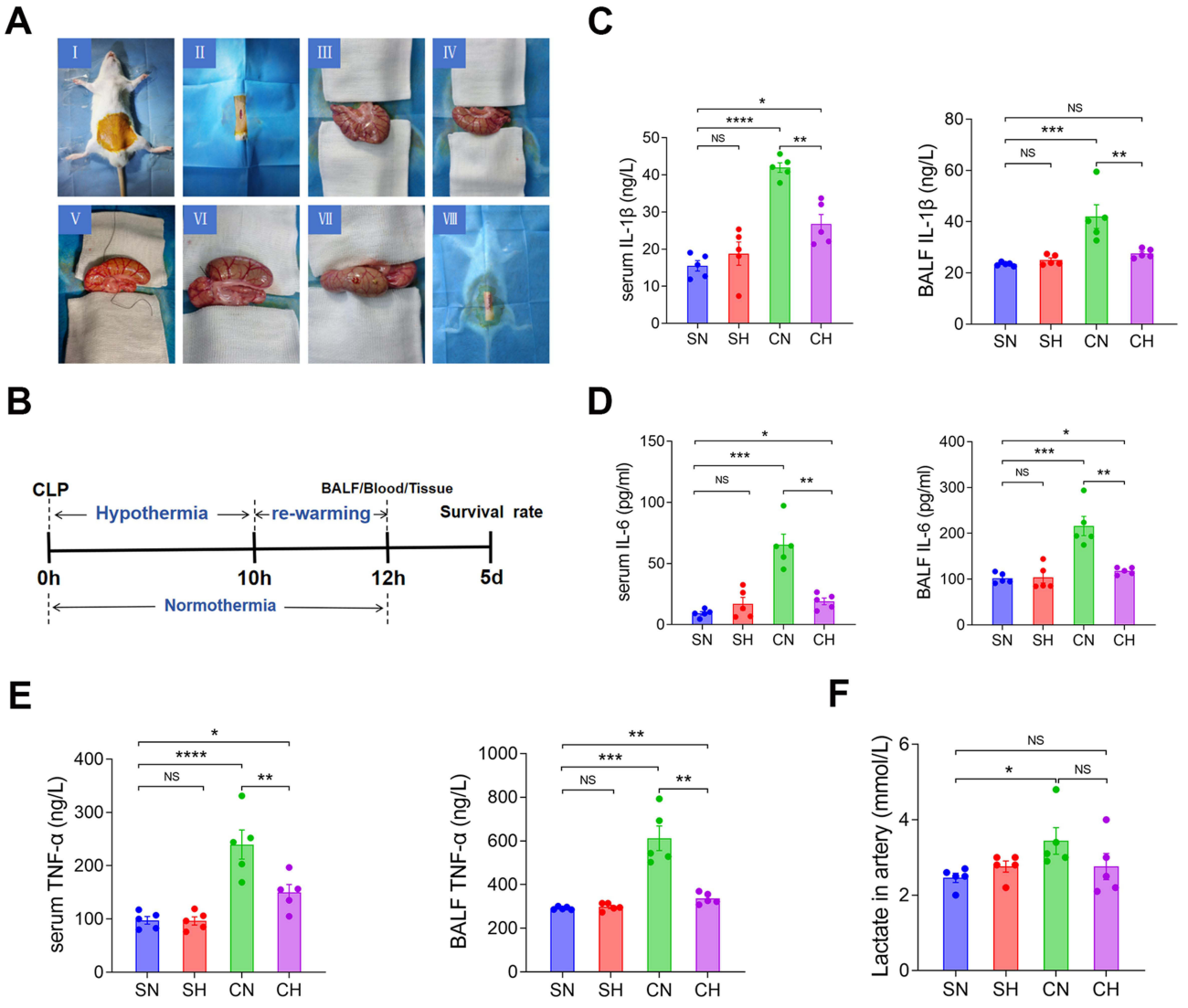

Figure 1 Moderate hypothermia decreased CLP-induced inflammatory cytokines and lactate levels. (**A**) Critical steps in the CLP procedure in rats.I.Disinfection of the abdominal region after shaving.II.A midline incision on the skin.III.Exposure of the cecum. IV-VI.Ligation of the cecum at designated positions. VII.Needle puncture of the cecum.VIII.The cecum was then repositioned within the abdominal cavity, and the incision was intermittently sutured in two layers with 4/0 sutures. (**B**) Schematic protocol of animal experiments. (**C–E**) Representative levels of IL-1β, IL-6, and TNF-α in serum and BALF. (**F**) Lactate concentration in blood gas analysis. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001.

Abbreviations: SN, sham-normothermia; SH, sham-hypothermia; CN, CLP-normothermia; CH, CLP-hypothermia; CLP, cecal ligation and puncture; BALF, bronchoalveolar lavage fluid. NS, no significance.

(32–33.9°C) for 10 hours. The rats were gradually rewarmed back to normothermia (36–38°C) at a rate of 1°C/h. Meanwhile, rats in the normothermia group were laid out on a thermostatic pad (maintained at 36–38°C) for 10 hours postoperatively. Anesthesia was sustained using 3% sodium pentobarbital (15 mg/kg) administered by intraperitoneal injection every hour during the temperature control period.

Experimental Design

Twenty male SD rats were randomly divided into the following four groups: sham-normothermia (SN), sham-hypothermia (SH), CLP-normothermia (CN), and CLP-hypothermia (CH), with five rats in each group. Twelve hours after CLP, the rats were sacrificed and samples were collected for analysis. The remaining 40 male SD rats were divided into groups as described above (10 per group) for survival analysis. Survival was recorded every hour on the first day after CLP and every two hours from the second to fifth days post-operation. Observations were terminated, and results were analyzed after five days (Figure 1B).

Arterial Blood Gas Analysis

Twelve hours after CLP or sham operation, 2.0 mL of blood was collected from the rats' abdominal aorta and analyzed using an ABL 90 blood gas analyzer (Radiometer Medical Devices Ltd., Denmark). The blood specimen was thoroughly mixed, and residual air was removed prior to testing. The sample was then processed according to the manufacturer's instructions to detect the partial pressure of arterial oxygen (PaO2), lactate concentration, and blood glucose level.

Determination of Protein Concentration in Bronchoalveolar Lavage Fluid (BALF)

Bronchoalveolar lavage fluid (BALF) was collected as previously described by Han et al.[37] Briefly, after the rats were anesthetized, the trachea was isolated and the right bronchus was ligated. A small incision was made in the trachea, allowing the lavage tube to enter the left bronchus. The lavage process was performed three times with 0.5 mL aliquots of pre-cooled saline into the left lung. The collected BALF was centrifuged at 3000 rpm for 10 minutes at 4°C, and the supernatant was stored at −80°C for further analysis. The protein content was determined using a BCA protein colorimetric assay kit (Elabscience, Wuhan, China).

Enzyme-Linked Immunosorbent Assay (ELISA)

Twelve hours after the CLP or sham operation, approximately 3 mL of blood was collected from the rat's abdominal aortic artery. The blood was allowed to clot for two hours at room temperature and then centrifuged at 3000 rpm for ten minutes at 4°C. The serum was separated and stored at −80°C for subsequent analysis. BALF was collected and processed in accordance with the previously described protocol. The concentrations of IL-1β, IL-6, and TNF-α in rat serum and BALF supernatant were detected using ELISA kits for the corresponding cytokines, according to the manufacturer's instructions. All the ELISA kits were purchased from Meimian (Jiangsu, China).

Lung Wet/Dry Weight Ratio

Pulmonary capillary permeability differences and tissue edema were estimated using the lung wet/dry weight ratio, a technique commonly used to assess experimental lung injury.[38] The upper lobe of the right lung was harvested and weighed (wet weight) and then dried at 65°C for 72 h (dry weight). The lung wet-to-dry weight ratio was calculated by dividing the wet weight by the dry weight.[39]

Hematoxylin and Eosin (HE) Staining

Rat lung tissues were isolated and fixed in 4% paraformaldehyde for 24 hours at room temperature before embedding in paraffin. The tissues were then sectioned into thin slices and subjected to hematoxylin-eosin (H&E) staining for histological examination. Lung injury was scored using a semiquantitative scoring system that considers alveolar and interstitial inflammation, edema, alveolar and interstitial hemorrhage, atelectasis, necrosis, and hyaline membrane formation.[40] Parameters of the tissue sections were scored according to the following scale: 0 (normal), 1 (mild), 2 (moderate), 3 (severe), and 4 (extremely severe). Each of the five parameters was scored individually, with a total possible score of 20 points.

Transmission Electron Microscopy (TEM)

Lung tissue specimens from rats were fixed in 3.0% glutaraldehyde and 1% osmium acid and then cut into ultrathin sections. The samples were double-stained with uranyl acetate and lead citrate, and then photographed using a Hitachi H-7650 transmission electron microscopy (Tokyo, Japan). Morphological changes such as decreased mitochondrial cristae and increased membrane density are primary distinguishing features of ferroptosis.[41] Hence, the mitochondrial ultrastructure of PMVECs in lung tissue was observed in different groups.

Immunohistochemistry

Paraffin-embedded lung tissue sections were deparaffinized, followed by hydration with graded ethanol. The deparaffinized tissue sections were heated in a microwave with sodium citrate buffer for antigen retrieval. The sections were then treated with 3% hydrogen peroxide to inactivate endogenous peroxidases, followed by blocking with bovine serum albumin to prevent nonspecific background staining. Subsequently, the primary antibody against GPX4 (1:200, GB114327, Servicebio) was added to the sections and incubated at 4°C overnight. Then, the sections were incubated with HRP-labeled goat anti-rabbit secondary antibody for 50 minutes at room temperature, followed by diaminobenzidine staining and counterstaining with hematoxylin. Finally, the slides were dehydrated with gradient alcohol, mounted, and analyzed under light microscopy.

Immunofluorescence

After the rat lung tissues were divided and pretreated, paraffin-embedded tissue sections were deparaffinized and rehydrated, followed by antigen retrieval and serum blocking. Then, the sections were incubated with a primary antibody against GPX4 at 4°C overnight in a humidified chamber. The next day, the slides were stained with CY3-conjugated secondary antibodies at room temperature for 50 minutes in the dark. Subsequently, 4',6-diamidino-2-phenylindole (DAPI) solution was used to stain the nuclei for 10 minutes, and the slides were sealed with a mounting solution containing an anti-fluorescence quencher. The results were observed and photographed using a fluorescence microimaging system (Axio Imager.A2, ZEISS, Germany).

Detection of Ferroptosis-Related Biomarkers (ROS, MDA, GSH, SOD, and Fe^{2+})

To image lipid peroxidation during ferroptosis propagation, serum ROS levels in the CLP and sham groups were detected using the BBoxiProbe O12 assay kit (BestBio, BB-475015). Further, the malondialdehyde (MDA) content in serum and BALF under different conditions was determined by the thiobarbituric acid method (Geruisi, G0109W). Right lung tissues were homogenized and centrifuged to obtain the supernatant. The GSH levels (Servicebio, G4305), superoxide dismutase (SOD) activity (Solarbio, BC5165), and Fe^{2+}content (Solarbio, BC5415) were measured according to the manufacturer's instructions.

Western Blot

The expression of PI3K, p-PI3K, Akt, p-Akt, GSK3β, p-GSK3β, Keap1, Nrf2, SLC7A11, and GPX4 was detected by Western blot. Rat lung tissue samples were fragmented with a homogenizer. Proteins were extracted from the tissue lysates and analyzed Western blot as previously described. Protein samples were separated by 10% or 12% SDS-PAGE and transferred onto PVDF membranes. Then, the membranes were blocked with 5% non-fat dried milk or 5% bovine serum albumin (BSA) in TBST for one hour at room temperature, followed by incubation with specific primary antibodies overnight at 4°C. The membranes were washed the next day and then incubated with the fluorescent secondary antibody for one hour at room temperature in the dark. Protein signals were recorded using a Li-Cor Odyssey Infrared Imaging System. ImageJ software was used to quantify the band densities. The relative expression levels of the target proteins were normalized using β-actin as an internal control.

Quantitative Real-Time Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted from rat lung tissues using the RNAeasy™ Animal RNA Isolation Kit with Spin Column (Beyotime, R0026) according to the manufacturer's instructions, and its quality was evaluated using the A260/A280 ratio. cDNA was synthesized using PrimeScript™ RT Master Mix (Takara, RR036A). Real-time quantitative PCR (RT-qPCR) was performed with the TB Green PCR Kit (Takara, RR820A), and relative mRNA levels were analyzed using β-actin as the reference gene. The primer sequences are listed in Table 1.

Table 1 Primer Sequences Used for the RT-qPCR Study

Gene	Forward Primers	Reverse Primers
β-actin	ACGGTCAGGTCATCACTATCG	GGCATAGAGGTCTTTACGGATG
Keap1	TGCCCCTGTGGTCAAAGTG	GGTTCGGTTACCGTCCTGC
Nrf2	ATGCCTTCCTCTGCTGCCATTAG	ACCGTGCCTTCAGTGTGCTTC
SLC7A11	TATGCTGAATTGGGTACGAGC	TATTACCAGCAGTTCCACCCA
GPX4	AGGCAGGAGCCAGGAAGTAATC	ACCACGCAGCCGTTCTTATC

Abbreviations: SA-ALI, sepsis-associated acute lung injury; PMVECs, pulmonary microvascular endothelial cells; Nrf2, nuclear factor erythroid 2-related factor 2; GPX4, glutathione peroxidase 4; Keap1, Kelch-like ECH-associated protein 1; GSK-3β, glycogen synthase kinase-3β.

Statistical Analysis

Statistical analysis was conducted using GraphPad Prism 9.0 software (GraphPad). Survival rates are presented as percentages, while other data are expressed as mean ± standard deviation (SD). Data were compared using unpaired two-tailed Student's *t*-test for two groups or one-way ANOVA followed by Tukey's correction for multiple groups. Log rank test was used to compare differences in survival. $P<0.05$ was considered to indicate statistical significance.

Results

Moderate hypothermia reduced the levels of CLP-induced inflammatory cytokines and arterial blood lactate

Sepsis is a complex clinical syndrome characterized by a systemic dysregulated host response to infection.[42] Hence, the extent of the inflammatory response in rats after CLP and moderate hypothermia treatment was assessed by detecting inflammatory cytokines in serum and BALF via ELISA. Results indicated that the IL-1β, IL-6, and TNF-α levels in both serum and BALF significantly increased after CLP treatment, but were downregulated in response to moderate hypothermia (Figure 1C–E). In addition, the effect of moderate hypothermia on arterial blood lactate levels was detected. Previous studies have shown that plasma lactate levels and lactate clearance are strongly associated with sepsis severity and mortality in clinical settings.[43–45] Our results revealed that arterial blood lactate concentration was significantly elevated in rats with CLP-induced sepsis, while moderate hypothermia had the opposite effect (Figure 1F). The above results indicate that moderate hypothermia could alleviate CLP-induced inflammatory response and reduce arterial blood lactate levels in rats.

Moderate hypothermia alleviated CLP-induced acute lung injury and improved the survival rate of rats with sepsis

To verify the protective effect of moderate hypothermia in a rat model of septic lung injury, first, we observed the histopathological changes in the rat lungs. HE results indicated that CLP induced alveolar collapse, interstitial edema, and neutrophil infiltration in the lung tissue, while moderate hypothermia significantly ameliorated the above injuries (Figure 2A and B). Second, we assessed the integrity of the lung barrier in the model rats. Results indicated that the lung wet/dry weight ratio and BALF protein concentration increased significantly following CLP, while moderate hypothermia elicited a reduction. Furthermore, arterial oxygenation was remarkably decreased after CLP treatment, while moderate hypothermia could improve arterial oxygenation in rats (Figure 2C–E). In addition, the effect of moderate hypothermia on survival outcomes was evaluated. Experimental results indicated that half of the rats died within 12 hours of CLP treatment and the 5-day mortality rate increased to 60%. In contrast, when treated with moderate hypothermia, the 12-hour mortality rate decreased to 30% and the 5-day survival rate increased from 40% to 70% (Figure 2F). The above results indicated that moderate hypothermia could alleviate CLP-induced acute lung injury and improve the survival rate of rats with sepsis.

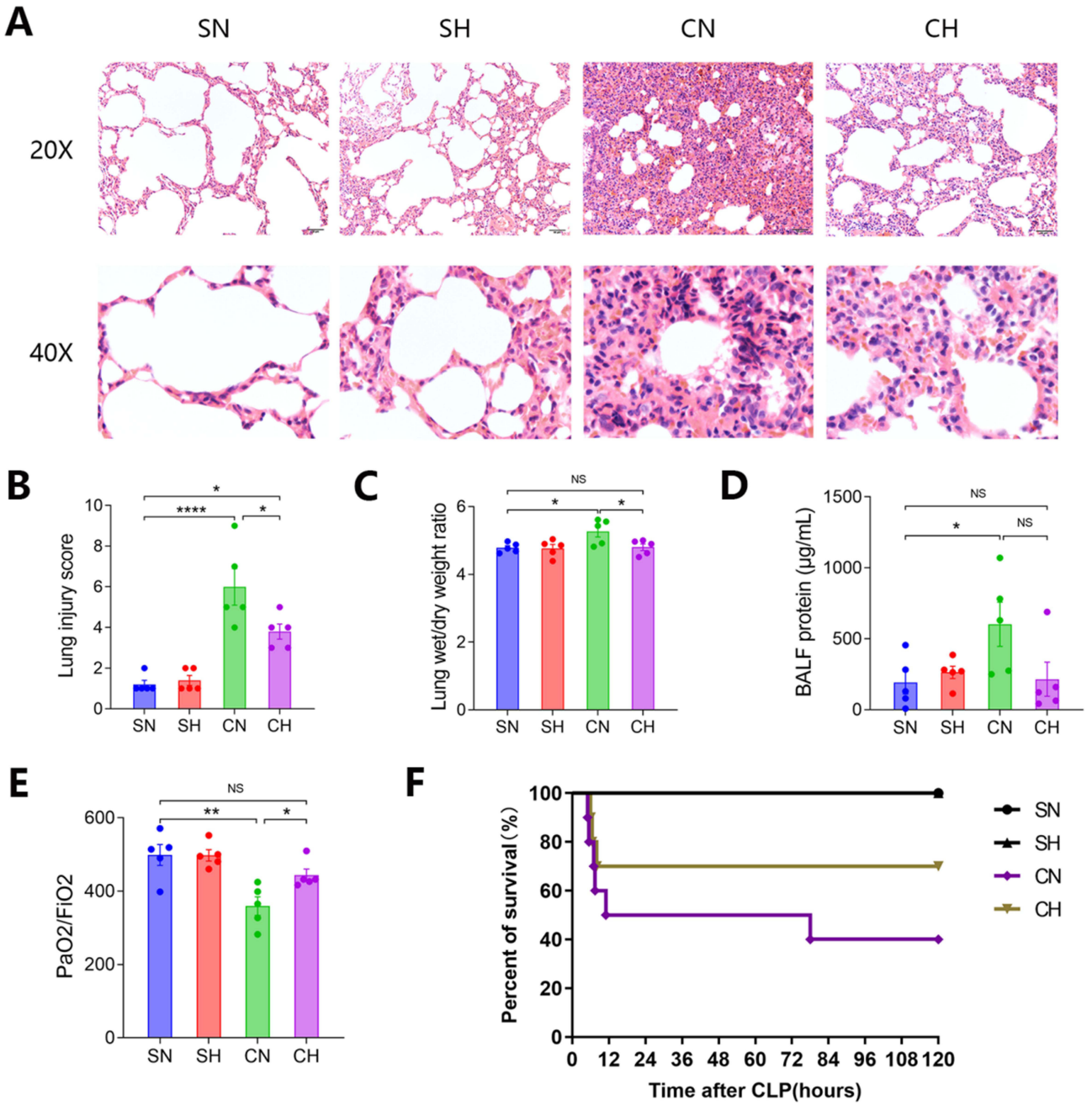

Figure 2 Moderate hypothermia alleviated CLP-induced lung injury and improved the survival rate of septic rats. (**A**) HE results showing histopathological changes in the lung tissues of SD rats subjected to CLP and treated with moderate hypothermia. Scale bar: 50 μm. (**B**) Quantification of the lung injury score in each group of rats. (**C-E**) Wet/dry ratio of the lung tissue, BALF protein concentration, and arterial oxygenation collected from CLP- and moderate hypothermia-treated SD rats. (**F**) Survivorship curves of SD rats from different groups; **P*<0.05, ***P*<0.01, and *****P*<0.0001.
Abbreviation: NS, no significance.

Moderate hypothermia reduced CLP-induced ROS and increased antioxidant ability in rats

The above results indicate that CLP increased the levels of inflammatory cytokines and caused lung tissue injury in rats, while moderate hypothermia alleviated the CLP-induced inflammatory response, ameliorated lung tissue injury, and reduced the death rate. Excessive ROS accumulation and impaired antioxidant capacity are critical factors contributing to acute lung injury in sepsis. Iron metabolism and the GSH antioxidant system are essential components of the ferroptosis execution and defense systems, respectively (Figure 3A).[6,9,46–49] To fully assess the effects of the CLP-induced inflammatory response and moderate hypothermia on oxidative stress and injury in lung tissues, GSH, SOD, Fe^{2+}, and

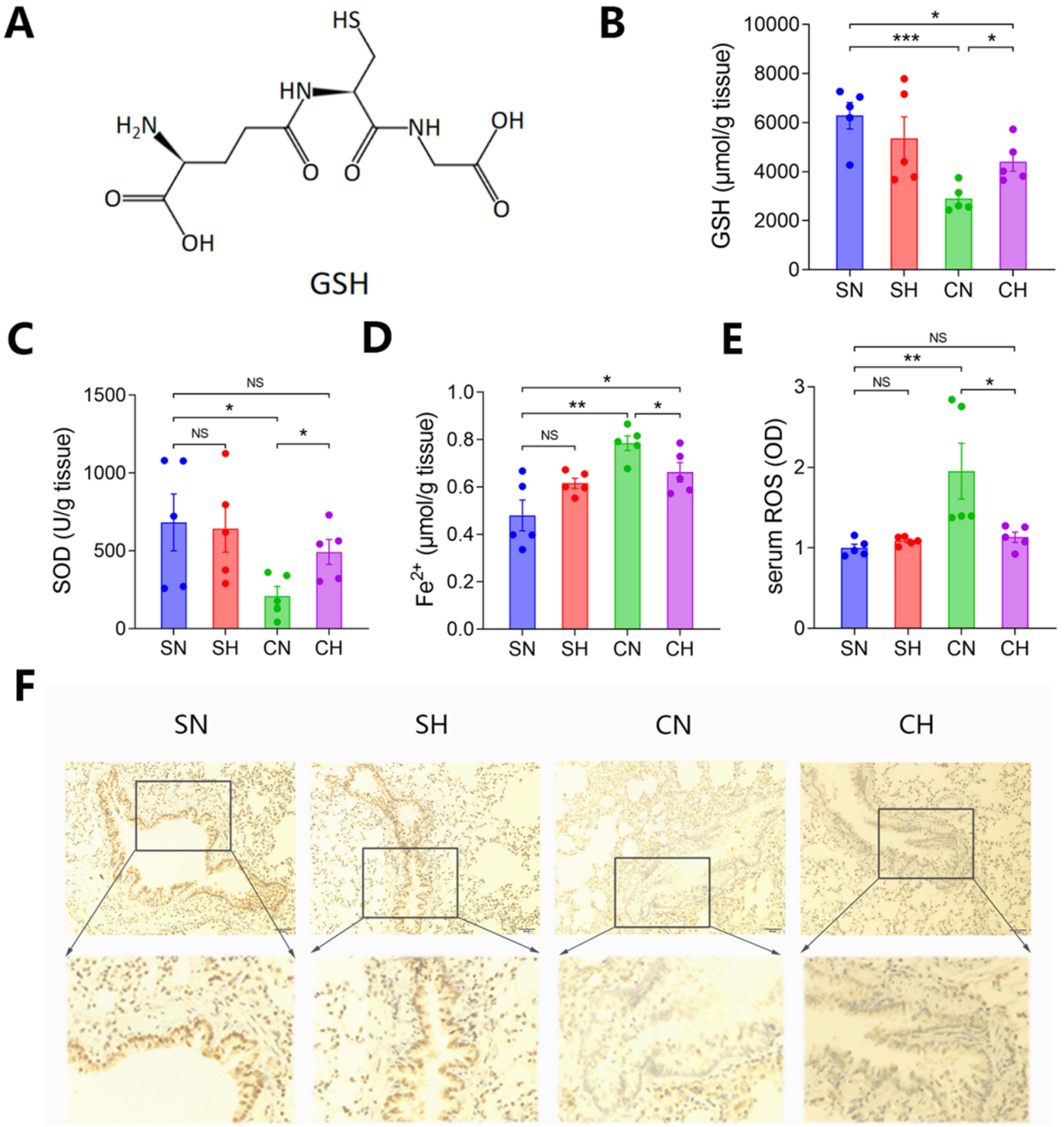

Figure 3 Moderate hypothermia reduced CLP-induced ROS and increased antioxidant capacity in rats. (**A**) Structure of glutathione(GSH). (**B–D**) GSH, SOD, and Fe^{2+}content in the lung tissue of CLP- and moderate hypothermia-treated SD rats. (**E**) The levels of serum reactive oxygen species (ROS) in rats. (**F**) GPX4 expression in the lung tissue of CLP- and moderate hypothermia-treated SD rats detected by immunohistochemical staining;, **P*<0.05, ***P*<0.01, and ****P*<0.001.
Abbreviation: NS, no significance.

ROS levels were detected. Results indicated that ROS levels and lung tissue Fe^{2+}content significantly increased after CLP treatment, while moderate hypothermia reduced their accumulation. Meanwhile, levels of antioxidants GSH and SOD were remarkably decreased following CLP, while moderate hypothermia restored their levels (Figure 3B–E). Furthermore, we used immunohistochemical staining to detect GPX4 expression in lung tissue. Results showed that GPX4-positive cells were significantly reduced in number after CLP, whereas their numbers increased after moderate hypothermia treatment (Figure 3F). In short, the above results indicate that following treatment with CLP, the defense

system against ferroptosis was significantly compromised, while the execution of ferroptosis was promoted, potentially exacerbating oxidative damage in rat lung tissues. In contrast, moderate hypothermia could increase antioxidant capacity and reduce CLP-induced ROS levels in rats.

Moderate hypothermia alleviated ferroptosis induced by excessive inflammation and oxidative stress in the lung tissue of septic rats

At the heart of ferroptosis is the lethal process of lipid peroxidation.[50] Malondialdehyde (MDA) is a primary component of lipid peroxides, and its concentration can be measured to assess the extent of lipid peroxidation, which is indicative of the state of ferroptosis (Figure 4A). Accordingly, MDA levels in the serum and BALF were detected among different groups of rats. Results indicated that MDA levels in the serum and BALF of rats were significantly increased following treatment with CLP, while the levels were remarkably decreased with moderate hypothermia treatment (Figure 4B and C). GPX4, an important intracellular antioxidant enzyme, negatively regulates ferroptosis by reducing lipid peroxidation.[51] Immunofluorescence staining results indicated that the expression of GPX4 was remarkably decreased in CLP-induced septic lung tissue and

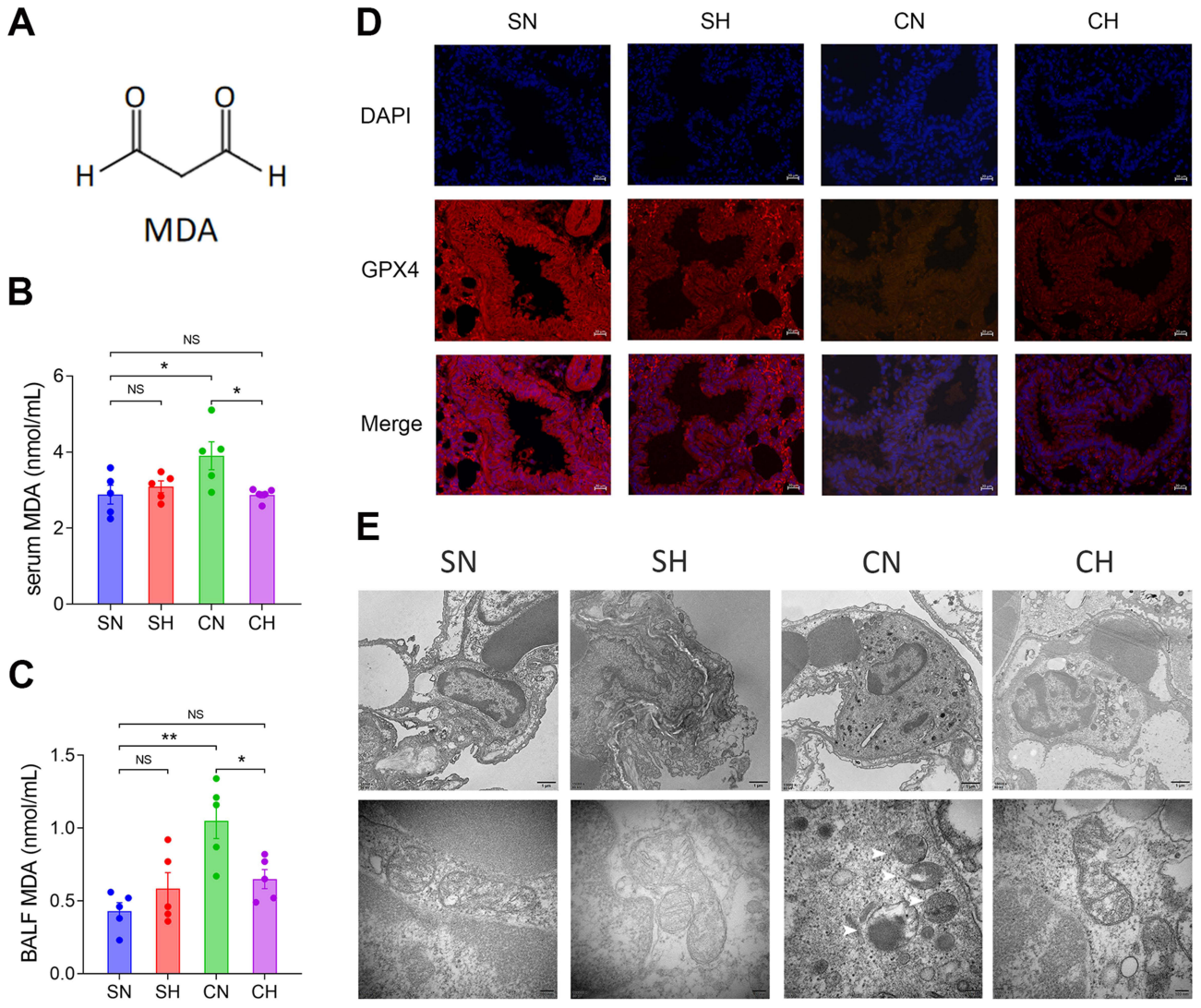

Figure 4 Moderate hypothermia inhibited ferroptosis induced by excessive inflammation and oxidative stress in the lung tissue of septic rats. (**A**) Structure of malondialdehyde (MDA). (**B** and **C**) MDA levels in the serum and BALF after different treatments. (**D**) GPX4 immunofluorescence staining results in lung tissue of CLP- and moderate hypothermia-treated SD rats. Blue: DAPI; Red: CY3. (**E**) Representative TEM images of PMVECs; single white arrowheads indicate shrunken mitochondria; **P*<0.05, and ***P*<0.01.

Abbreviation: NS, no significance.

slightly upregulated in rats treated with moderate hypothermia (Figure 4D). To further investigate the effects of CLP and moderate hypothermia on ferroptosis, we employed TEM for visualization. Interestingly, TEM results indicated that PMVECs in CLP-treated rat lung tissues exhibited cellular morphological characteristics specific to ferroptosis, such as reduced mitochondrial volume, increased membrane density, and decreased or absent mitochondrial cristae. In contrast, moderate hypothermia significantly ameliorated the above injuries (Figure 4E). These findings indicate that moderate hypothermia could alleviate excessive inflammation and oxidative stress-induced ferroptosis by increasing GPX4 antioxidant enzyme activity and reducing lipid peroxidation levels.

Moderate hypothermia upregulated GPX4 expression in lung tissue via the Keap1/Nrf2/SLC7A11 signaling pathway

The Keap1/Nrf2 signaling pathway is a classical and crucial pathways for cellular defense and survival, alleviating oxidative injury by regulating downstream antioxidant genes such as *SLC7A11* and *GPX4*.[52] Western blot and RT-qPCR results indicated

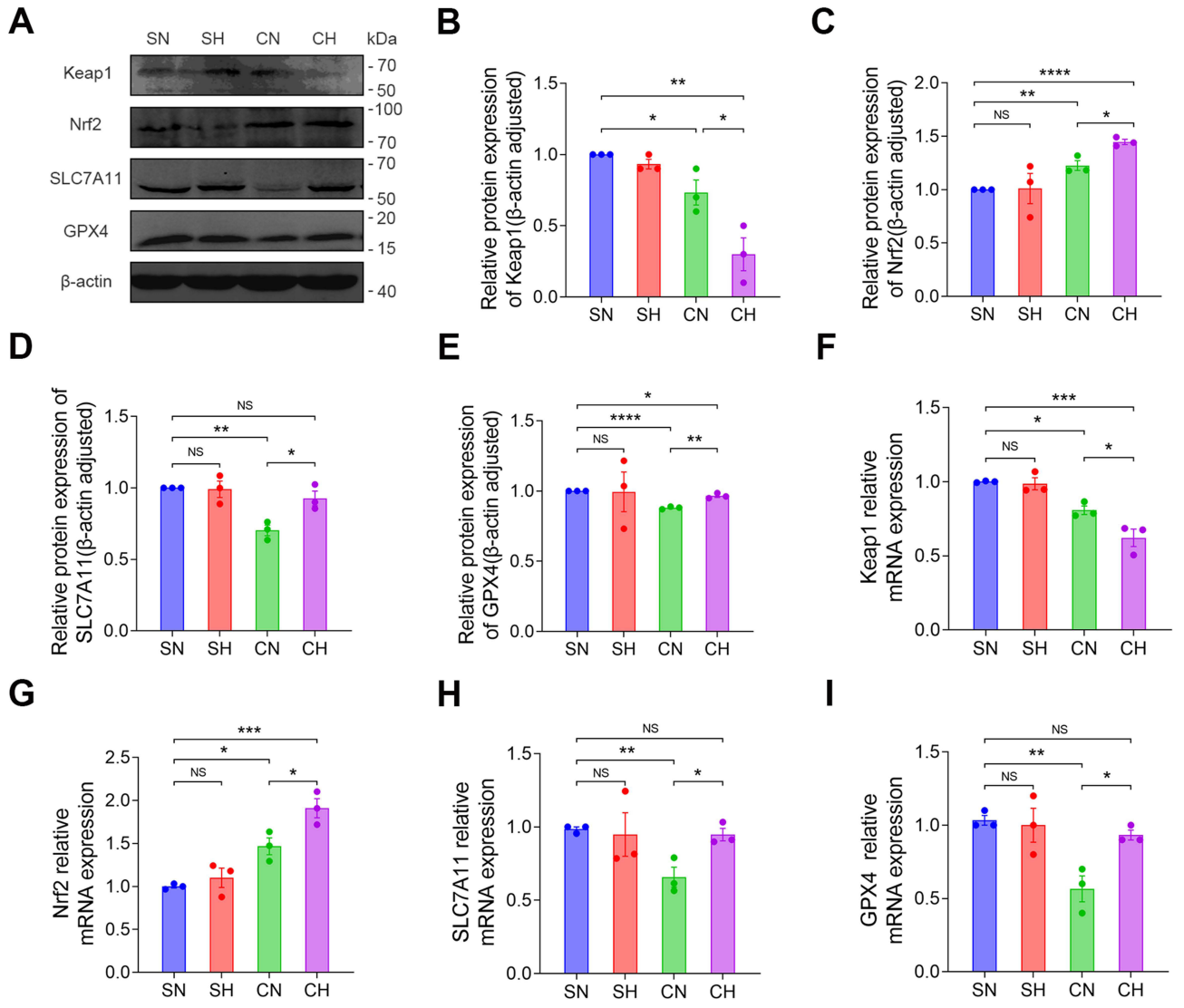

Figure 5 Western blot and RT-qPCR results for vital signaling molecules in the Keap1/Nrf2/ SLC7A11/GPX4 axis. (**A**) The protein expression of Keap1, Nrf2, SLC7A11, and GPX4 in lung tissue of CLP- and moderate hypothermia-treated SD rats was detected by Western blot. (**B–E**) Quantitative analyses of protein expression were performed using ImageJ. (**F–I**) mRNA expression of Keap1, Nrf2, SLC7A11, and GPX4 in lung tissue of rats from different groups were detected by RT-qPCR; **P*<0.05, ***P*<0.01, ****P*<0.001, and *****P*<0.0001.

Abbreviation: NS, no significance.

that the expression of Keap1, a negative regulator of Nrf2, was slightly reduced in CLP-induced sepsis lung tissue and remarkably decreased following moderate hypothermia (Figure 5A–F). In contrast, Nrf2 protein expression was slightly upregulated in CLP-induced sepsis lung tissue and remarkably increased upon treatment with moderate hypothermia (Figure 5A–C). RT-qPCR results also revealed that the expression levels of *Nrf2* mRNA in the lung tissues of septic rats significantly increased after moderate hypothermia treatment (Figure 5G). Nrf2 upregulation and translocation into the nucleus can activate the downstream target genes.[23–25,53] Western blot and RT-qPCR results indicated that the expression of SLC7A11 and GPX4 was significantly reduced in CLP-induced sepsis lung tissue and remarkably increased after moderate hypothermia treatment (Figure 5A– I). Taken together, the above results indicate that moderate hypothermia could upregulate GPX4 expression via the Keap1/Nrf2/SLC7A11 signaling pathway.

Moderate hypothermia increased Nrf2 protein levels via the PI3K/Akt/GSK3β signaling pathway in CLP-induced acute lung injury

The PI3K/AKT pathway plays a significant role in cellular proliferation and survival by regulating the activity of downstream molecules such as mTOR, GSK-3β, and FoxO1.[54,55] During oxidative stress, glucose-stimulated insulin secretion (GSIS) occurs in pancreatic β-cells. ROS, such as H_2O_2 derived from glucose metabolism, promote the phosphorylation and activation of receptor tyrosine sites.[56,57] Binding of the activated receptor to insulin activates PI3K, which converts phosphatidylinositol-4,5-bisphosphate (PIP2) into phosphatidylinositol-3,4,5-trisphosphate (PIP3). PIP3 binds to phosphoinositide-dependent protein kinase-1 (PDK1) and Akt. Akt is subsequently fully activated after two phosphorylation events. First, PDK1 weakly phosphorylates Akt at Thr-308, which, in turn, triggers a second, stronger phosphorylation of Akt at Ser-473 by the mammalian target of rapamycin complex 2 (mTORC2).[58,59] Western blot results indicated that the protein levels of p-Tyr467/Tyr199 PI3K and p-Ser473 Akt were slightly increased in CLP-induced rat lung tissues and significantly upregulated upon treatment with moderate hypothermia (Figure 6A–F). The activity of GSK-3β can be inhibited by Akt-mediated phosphorylation at Ser9.[60] Interestingly, treatment with moderate hypothermia increased the expression levels of p-Ser9 GSK-3β, thereby downregulating GSK-3β protein activity. This process can effectively promote glycogen synthesis and negatively regulate blood glucose through feedback mechanisms. Meanwhile, owing to the inhibition of GSK-3β and the subsequent loss of repression of Nrf2 by β-TrCP, the ubiquitylation and degradation of Nrf2 were reduced (Figure 6A– I). The above results indicate that moderate hypothermia could upregulate Nrf2 protein levels via the PI3K/Akt/GSK3β signaling pathway, which is involved in the regulation of ferroptosis.

Discussion

Our research demonstrated that moderate hypothermia can attenuate SA-ALI through inhibiting ferroptosis induced by excessive inflammation and oxidative stress via the Keap1/Nrf2/SLC7A11/GPX4 signaling pathway. In addition, the activation of the glucose feedback regulatory pathway PI3K/Akt/GSK3β may have partially contributed to the protective effects of moderate hypothermia in septic rats. These findings offer a new perspective for the investigation of SA-ALI.

ALI is a severe complication that significantly contributes to the high mortality rate associated with sepsis. However, treatment options for SA-ALI are limited, relying primarily on antibiotics, mechanical ventilation, and supportive care.[61] Therefore, there is an urgent need to explore more effective therapeutic measures, with hypothermia receiving increasing attention in recent years. The strongest data supporting the use of hypothermia exist in comatose survivors of out-of-hospital cardiac arrest who have undergone cardiopulmonary resuscitation.[62] However, there is still controversy in the medical community regarding the use of hypothermia in sepsis. Some scholars believe that hypothermia can exert several effects that may mitigate cardiopulmonary dysfunction and vasoplegia during septic shock.[28,63] In contrast, other scholars have found that hypothermia does not reduce mortality in patients with septic shock and ventilator-dependent respiratory failure, and may even delay the recovery of several vital organ functions.[33] Hence, given the conflicting results regarding the use of hypothermia as a treatment for sepsis, further research is needed to determine its role and the regulatory mechanisms involved. Based on data from the previous study, we selected a target temperature range of 32–33.9°C for 10 hours. Preliminary results indicated that moderate hypothermia could reduce inflammatory cytokine and lactate levels,

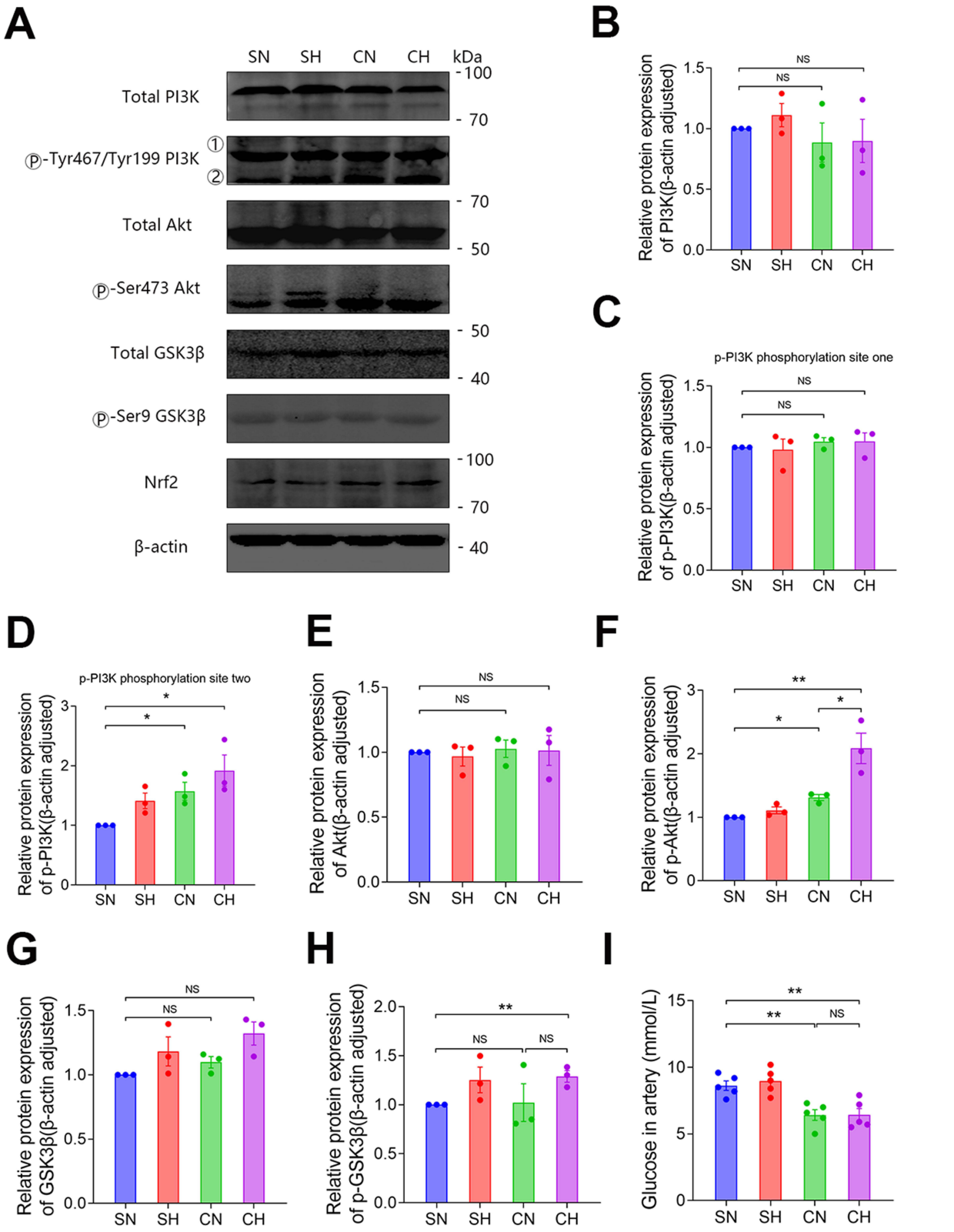

Figure 6 Expression levels of key components in the PI3K/Akt/GSK3β/Nrf2 signaling pathway and results of the rat arterial blood glucose assay. (**A**) PI3K, p-PI3K, Akt, p-Akt, GSK3β, p-GSK3β, and Nrf2 expression in lung tissue of CLP- and moderate hypothermia-treated SD rats was detected by Western blot. (**B–H**) Quantitative analyses of protein expression. (**I**) Results of blood glucose measured twelve hours post-op; *P<0.05, and **P<0.01.
Abbreviation: NS, no significance.

alleviate CLP-induced lung tissue injury, and improve the survival rate of septic rats. In fact, moderate hypothermia is more effective when used in the early stages rather than terminal stages of sepsis, serving as a treatment rather than as a means of resuscitation.

Ferroptosis is a form of iron-dependent regulated cell death triggered by the excessive accumulation of lipid ROS.[41] A growing body of research confirms the close relationship between ferroptosis and sepsis.[12–14] Hence, some scholars have proposed the concept of iron-targeted "nutritional immunity". Iron is a key trace element for microbial survival and has been shown to increase bacterial virulence and accelerate pathogen growth.[64] During sepsis, iron metabolism is altered, characterized by increased intracellular iron transport and uptake and decreased iron export. The sequestration of iron within cells limits its utilization by circulating pathogens and provides defensive protection.[6] However, as sepsis progresses, intracellular iron overload occurs due to the accumulation of lysosome-phagocytosed labile iron derived from extracellular sources as well as the further catabolism of ferritin and iron-rich intracellular organelles (eg, mitochondria). These redox-active iron pools directly catalyze the formation of destructive free radicals through the Fenton reaction, leading to oxidative injury and ferroptosis.[47] Ferroptosis releases iron for bacterial multiplication and spread, promoting the onset and development of sepsis and thereby exacerbating immune dysfunction, which may eventually cause multi-organ failure. Our experimental data support the above viewpoints and show that moderate hypothermia could alleviate acute lung injury in sepsis by enhancing antioxidant enzyme activity and mitigating ferroptosis.

Nrf2 is a modular protein containing seven highly conserved Nrf2-ECH homology domains (Neh1-7), each with a distinct function (Figure 7) NRF2[18,65]. It is now recognized that Keap1 functions as a substrate adaptor protein for the Cul3 RING-box 1 (RBX1) E3 ubiquitin ligase complex (called CRL^{Keap1}), which is responsible for mediating the ubiquitylation and degradation of Nrf2 under normal homeostatic conditions (Figure 7) KEAP1[66–69]. Experimental results demonstrate that CLP treatment increased the levels of inflammatory cytokines and ROS production in rats. The cysteine residues in Keap1 are modified by ROS during oxidative stress conditions, leading to reduced CRL^{Keap1} activity. As a result, Nrf2 rapidly accumulates and translocates to the nucleus, where it activates downstream target gene expression and participates in the regulation of ferroptosis. However, sustained high levels of oxidative stress may overwhelm the Nrf2 antioxidant system, failing to protect cells from oxidative damage and death.[57] Our results show that moderate hypothermia in CLP-treated rat lung tissues can regulate programmed cell death by affecting the stability of Nrf2 protein.

GSK-3β is another significant negative regulator of Nrf2.[26] The Neh6 structural domain of Nrf2 is a serine-rich region containing two conserved peptide motifs, DSGIS and DSAPGS. A GSK-3β phosphorylation site is present in the DSGIS motif, which is one of the β-TrCP recognition sites. The substrate receptor β-TrCP is an F-box-containing protein that utilizes its C-terminal WD40 protein-protein interaction domain to bind substrates and its F-box module to bind the Skp1-Cullin-1-F-box protein (SCF) E3 ubiquitin ligase complex.[70] The SCF E3 ubiquitin ligase complex formed by β-TrCP is called $SCF^{\beta\text{-TrCP}}$. The serine residues in the DSGIS motif are phosphorylated by GSK3β to promote recognition by $SCF^{\beta\text{-TrCP}}$, thereby facilitating the recruitment of the SCF ubiquitin ligase complex and the proteasomal degradation of Nrf2 (Figure 7) GSK3β[15,18]. In this study, we found that the constitutive activity of GSK-3β is inhibited by Akt phosphorylation; thus, it is apparent that PI3K stimulation prevents the antagonism of Nrf2 by GSK-3β. Notably, moderate hypothermia may protect the lungs from sepsis via a PI3K/Akt/GSK-3β-dependent mechanism.

The above results indicate that hypothermia is a highly promising therapeutic strategy. Hypothermia is the first treatment demonstrated to reverse post-ischemic injury in clinical settings. Apart from lowering metabolism and reducing oxygen consumption, these therapeutic effects of hypothermia involve various protective mechanisms, including attenuating the immune-inflammatory response, reducing free radical production, decreasing vascular permeability, protecting mitochondrial function, and delaying the onset of acidosis.[71] Multiple cellular mechanisms in organisms are temperature-dependent, including endothelial cell dysfunction, ROS production, and apoptosis.[27] One consequence of hypothermia is decreased insulin secretion, which can lead to hyperglycemia. The ROS produced by glucose in a controlled manner participate in intercellular signal transduction.[57,71] Another consequence of hypothermia is the significant reduction in the amount of free radicals produced, allowing endogenous antioxidative mechanisms to better cope with the free radicals being released. This process prevents or substantially mitigates oxidative injury and promotes cellular self-repair and recovery.[72–75] Inflammatory response and oxidative stress are two biological events that are

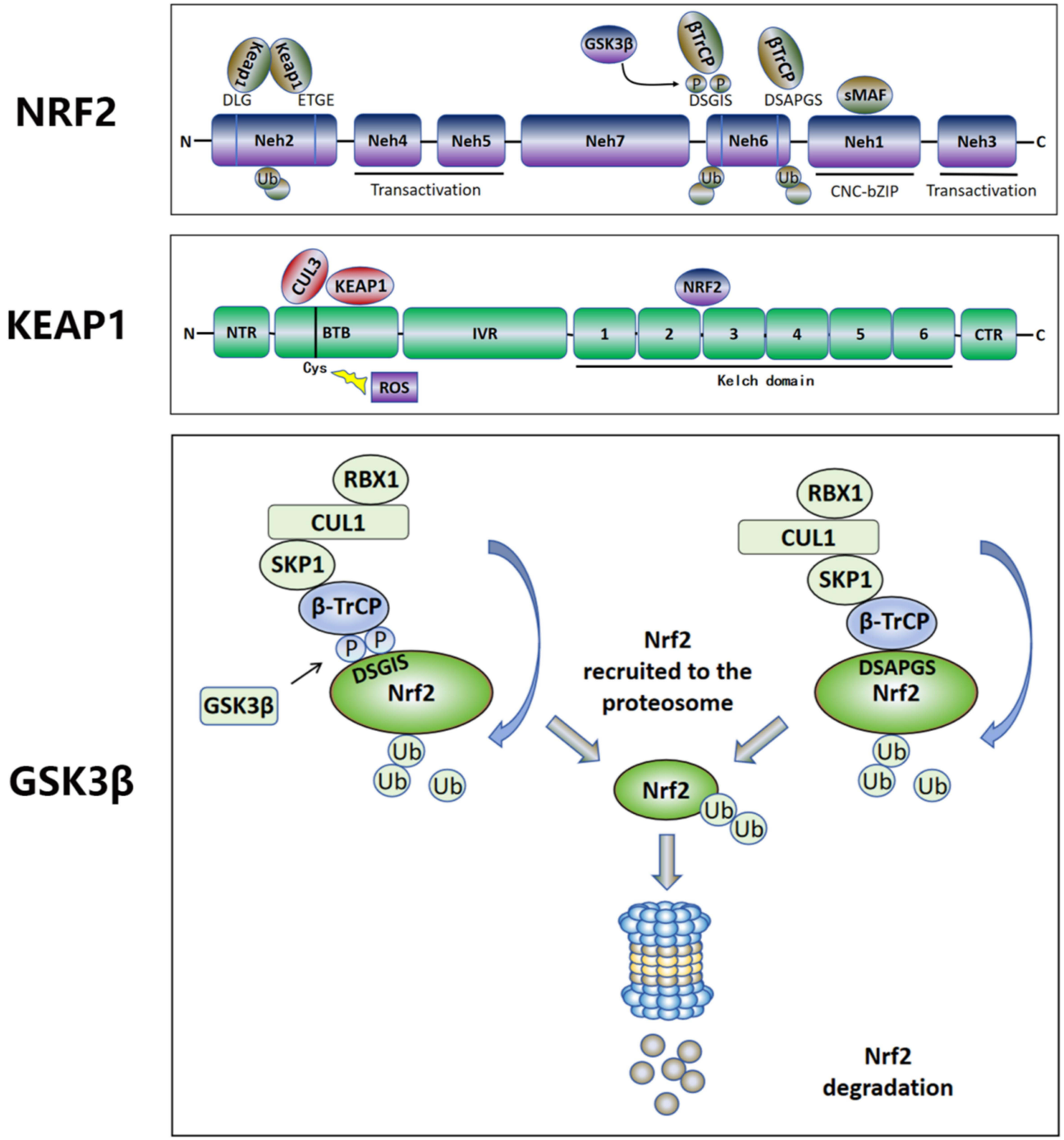

Figure 7 Suppression of NRF2 activity by KEAP1 and β-TrCP.

relatively independent but closely linked in acute lung injury in sepsis. Our previous studies have shown that hypothermia can attenuate septic lung injury in rat models by inhibiting the inflammatory response.[34,35] In this study, our findings demonstrated that the antioxidant effects of moderate hypothermia on CLP-induced acute lung injury can be attributed to the activation of the transcription factor Nrf2 via the ROS/Keap1 and PI3K/Akt/GSK3β signaling pathways (Figure 8).

The study has certain limitations. The severity of sepsis in our CLP model may exceed that of cases typically reported in clinical trials. Additionally, the model did not include mechanical ventilation or antibiotics, whose absence may have increased mortality compared with that in clinical settings. Despite these limitations, the manifestations of organ

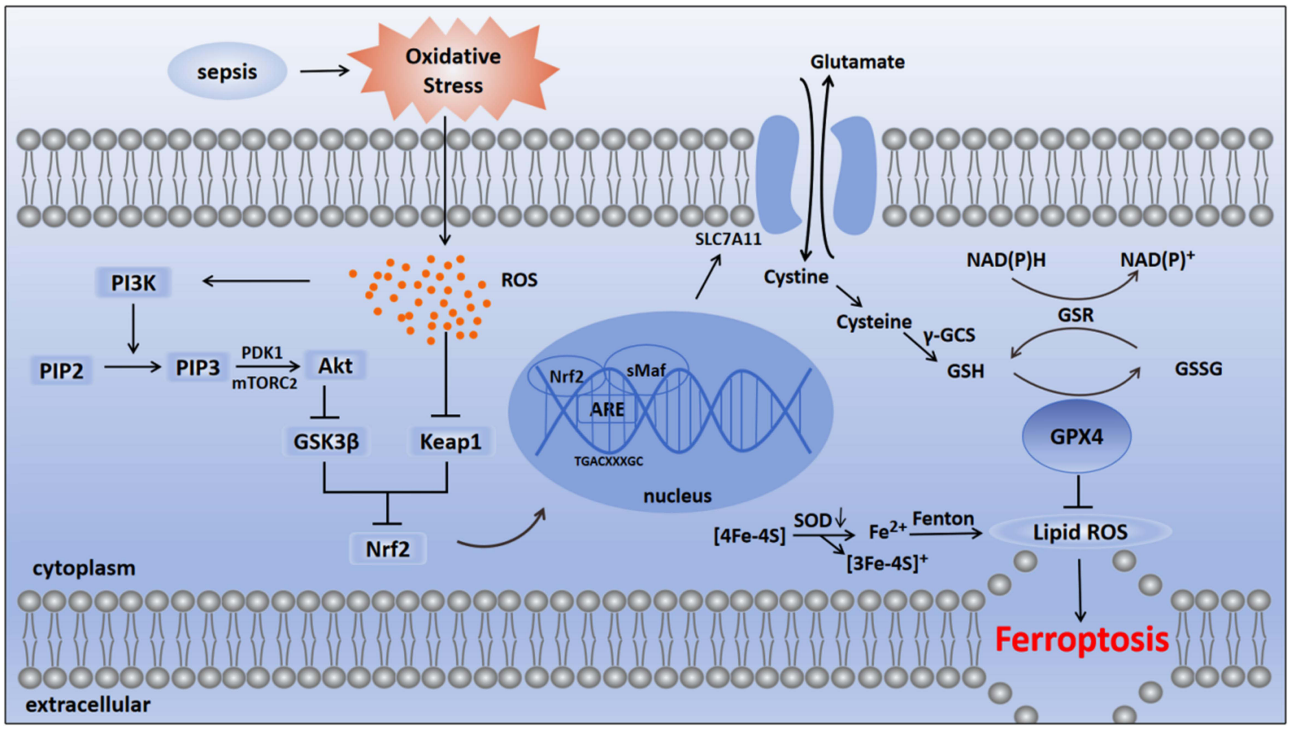

Figure 8 Cellular iron metabolism and antioxidant systems. Moderate hypothermia can alleviate excessive inflammation and oxidative stress induced ferroptosis by upregulating Nrf2/SLC7A11/ GPX4 expression via the ROS/Keap1 and PI3K/Akt/GSK3β axis, thereby ameliorating acute lung injury in sepsis.

dysfunction in our model still generalize to those of human SA-ALI, suggesting that the biological findings may be applicable to the human form of the disease. In terms of using hypothermia protocols in clinical trials, we would offer the following suggestions: a) the benefits of hypothermia are general and should be considered as an adjunct measure, to be used in conjunction with other conventional treatment regimens rather than as a substitute; and b) the optimal target temperature and duration may vary depending on the severity of sepsis or organ injury.

Conclusion

In conclusion, our results indicate that moderate hypothermia exerted a protective effect in the SA-ALI rat model. Moderate hypothermia may partially ameliorate lung injury by regulating the Keap1/GSK3β/Nrf2/GPX4 signaling pathway, inhibiting excessive inflammation and oxidative stress, and reducing ferroptosis. In addition, our study reveals a novel potential therapeutic mechanism by which moderate hypothermia could protect the lung by suppressing excessive inflammation, oxidative stress, and ferroptosis, providing a new avenue for the development of therapeutic strategies against SA-ALI. Increasing numbers of studies have demonstrated that oxidative stress is closely related to ferroptosis, and future research should aim to explore the mechanism of action underlying their relationship in SA-ALI.

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Disclosure

The authors report no potential conflicts of interest in this work.

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