Preconditioning with rHMGB1 ameliorates lung ischemia-reperfusion injury through inhibiting alveolar macrophages pyroptosis via Keap1/Nrf-2/HO-1 signal pathway

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Abstract

Background Lung ischemia-reperfusion injury (LIRI) is a common and complex pathophysiological process that can lead to poor patient outcomes. Inflammasome-dependent macrophage pyroptosis contributes to organ damage caused by ischemia-reperfusion (I/R). Oxidative stress reaction and antioxidant enzymes also play an important role in LIRI. This experiment was conducted to investigate whether preconditioning with rHMGB1 could ameliorate LIRI and explore the mechanisms of its protective effect in a lung I/R mice model.

Methods Adult male mice were anesthetized and the left hilus pulmonis was clamped for 60 min, followed by 120 min of reperfusion. rHMGB1 was performed by intraperitoneal injection at 2 h before anesthesia. Brusatol (Nrf-2 antagonist) was given intraperitoneally every other day for a total of five times before surgery. Measurements of pathohistological lung tissue damage, pulmonary wet/dry (W/D) ratios, inflammatory mediators were performed to assess the extent of lung injury after I/R. Alveolar macrophages (AMs) pyroptosis were evaluated by LDH release, caspase-1 expression in flow cytometry, GSDMD expression in immunofluorescent staining. Measurement of the products of oxidative Stress (ROS, MDA, 15-F2t-Isoprostane) and the antioxidant enzymes (SOD, GSH-PX, CAT) were performed.

Results Preconditioning with rHMGB1 significantly ameliorated I/R-induced lung injury through measuring the morphology, wet/dry weight ratio, the expressions of IL-1β, IL-6, NF-κB, HMGB1 in lung tissue. rHMGB1 preconditioning remarkably alleviated AMs pyroptosis induced by lung I/R. rHMGB1 preconditioning significantly reduced oxidative stress and restored the activity of antioxidative enzymes. In addition, rHMGB1 preconditioning mediated the activity of Keap1/Nrf-2/HO-1 pathway in LIRI. Furthermore, inhibiting Keap1/Nrf-2/HO-1 pathway through brusatol administration could aggravate lung injury.
tissue damage and inflammatory response after lung I/R. Also, brusatol administration could suppress the antioxidant and anti-pyroptosis effects of rHMGB1 preconditioning in LIRI.

Conclusions rHMGB1 preconditioning protects against LIRI through suppressing AMs pyroptosis. The mechanism is partially explained by inhibiting oxidative stress and improving the activity of antioxidative enzymes via Keap1/Nrf-2/HO-1 pathway.

Background

Lung ischemia-reperfusion injury (LIRI) is a complex pathophysiological process resulted from various clinical conditions, such as cardiac arrest, trauma, pulmonary thrombosis, lung transplantation, cardiopulmonary bypass surgery [1–2]. Respiration failure induced by LIRI is a vital risk factor for acute lung injury (ALI) or acute respiratory distress syndrome (ARDS) and indicates worse patient outcomes [3]. However, the potential molecular mechanism of LIRI remains unclear, and the effective methods of prevention and treatment are still lacking.

Recent studies suggest that sterile inflammation resulted from innate immune response plays an important role in tissue damage after organ I/R [4]. The magnitude of inflammation depends on the activation of pattern recognition receptors (PRRs). Alveolar macrophages (AMs) are the primary resident immune cells and play a pivotal role in innate immune response. AMs can be activated by the recognition of damage-associated molecular patterns (DAMPs) via PRRs [5–6]. During the early stage of lung I/R, the resident AMs have the function of controlling inflammatory factors. When the host response cannot re-establish homeostasis, the inflammatory response expands uncontrollably and further progresses to ALI and/or ARDS [7–8].

Pyroptosis is a programmed cell death accompanied with the release of inflammatory factors [9]. It is known that gasdermin-D (GSDMD) mediates pyroptosis after its cleavage
through caspase-1 canonical pathway or caspase-11 noncanonical pathway [10-12].
Recent studies have shown that inflammasome-dependent macrophage pyroptosis contributes to organ damage caused by I/R [13-14]. However, the exactly mechanism of activating or controlling AMs pyroptosis in LIRI remains unclear.
High mobility group protein 1 (HMGB1), as a kind of damage signal molecule, can be recognized and has closely related to initiate the innate inflammatory response [15-16]. Studies suggest that preconditioning with recombinant HMGB1 (rHMGB1) can protect against myocardial, kidney, and cerebral I/R injury [17-19]. In addition, HMGB1 has the function of activating reactive oxygen species and induce oxidative stress [20-21].
Oxidative stress triggers subsequent excess inflammatory factors accumulation and cascades of inflammatory response, thus resulting in severe lung tissue damage [22-23]. Therefore, preconditioning with rHMGB1 to protect the lung against oxidative stress might be an feasible method for ameliorating LIRI.
Numerous studies have demonstrated that Keap1/Nrf-2/HO-1 signal pathway has a close relationship with the oxidation and antioxidation processes [24-25]. NF-E2-related factor-2 (Nrf2) and Kelch-like ECH associating protein 1 (Keap1) are bound together normally. When stimulated, Nrf2 is degraded in the Keap1-dependent pathway and activates the production of heme-oxygenase 1 (HO-1). Activation of Keap1/Nrf-2/HO-1 pathway may protect against oxidative stress and apoptosis under various pathological conditions, including organ I/R injury. Recent study further showed that suppressing pyroptosis via the activation of the Nrf-2/HO-1 pathway could ameliorate renal I/R injury [26]. Therefore, this study aimed to determine whether preconditioning with rHMGB1 can ameliorate LIRI through inhibiting AMs pyroptosis via Keap1/Nrf-2/HO-1 signal pathway.

Materials And Methods
Animals

Adult male BALB/C mice (30 ± 5 g, Animal Centre, Guangxi Medical University, China) were used in our experiments. The animal protocol was authorized by the Institutional Animal Care and Use Committee of Guangxi Medical University (Nanning, China). All animal experiments were complied with the animal's guidelines of the Guangxi Medical University Institutional Animal Care and Use Committee.

Animal model of LIRI

LIRI animal model was processed as previously reported [8]. Briefly, an intraperitoneal injection of was adopted to anesthetize the animals. Pentobarbital (50 mg/kg) was used for anesthetizing the mice by intraperitoneal injection. Small animal ventilator (RSP1002-type) with a breathing frequency 80 breaths/min and a respiratory ratio (I/R) of 1:1 was performed for mechanical ventilation. The tidal volume was set at 10 ml/kg and the fraction of inspired oxygen was set at 100%. Lung collapse and expansion were closely watched. The mice in I/R group were underwent thoracotomy, and then occluded the left hilus pulmonis by a microvascular clamp for 60 minutes (including pulmonary artery, vein, and bronchi). Subsequently, 120 minutes of reperfusion was performed before closing thoracic incision. Two hours later, mice were sacrificed using cervical vertebra dislocation. The lower portions of the left lung were used for measurement. There was no mouse mortality during the whole experiments.

Treatment protocols

rHMGB1 was performed at 20 ug per mouse (Abcam, USA) by intraperitoneal injection at 2 h before anesthesia. The rHMGB1 dose was chosen based on previous study [18]. Endotoxin of the reagent was tested and contained < 0.01 EU/mg. Thus, the use of rHMGB1 can be considered endotoxin free. Brusatol, a Nrf-2 antagonist (0.4 mg/kg, BOC Science,
USA) was given intraperitoneally every other day for a total of five times before surgery. The usages of Brusatol based on previous study [22].

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

As we described previously [8, 27], 4% paraformaldehyde was used for fixing the lower portions of the left lung tissues, and then the tissues were embedded in paraffin. Sections slicing from the paraffin were evaluated by experienced technicians who were blinded to the experimental groups. The slices (4 microns) were waxed off by hydration and xylene, stained 5 min by hematoxylin, differentiated 30 sec by hydrochloric acid ethanol, soaked 15 min in water, stained 2 min by eosin. After conventional dehydration, transparent and sealing, H&E staining were completed.

**Microscopy and histology scoring of lung injury**

As we described previously [8], H&E stained paraffin slices were observed through light microscopy. An experienced technician who was blinded to the groups was assigned to assess the levels of lung injury by a scoring system. 10 fields were assessed at 200 × total magnification for each mouse. Lung injury scoring was performed as following. Briefly, the first criterion was aggregation or infiltration of inflammatory cells in vessel walls or air space: 1 = only wall, 2 = rarely cells in air space, 3 = intermediate, 4 = severe (air space congested). Second criterion was hyaline membrane formation and interstitial congestion: 1 = normal lung, 2 = moderate (> 25% of lung section), 3 = intermediate (25%-50% of lung section), 4 = severe (> 50% of lung section). Third criterion was hemorrhage: 0 = absent, 1 = present.

**Determination of wet/dry ratio**

As we described previously [8], pulmonary wet/dry (W/D) ratios were measured as an index of pulmonary edema and congestion. After mice were killed, the lower portions of
the left lung was immediately weighed and then dried to a constant weight at 60°C for 24 h.

Pulmonary W/D ratio = wet weight/dry weight.

**Collection of BALF and alveolar macrophages**

AMs were isolated as we described previously [28]. In brief, the lungs were flushed once with 5 ml of cold phosphate buffered saline through the cannulated trachea (Dulbecco’s PBS, Gibco BRL, Grand Island, USA) to collect bronchoalveolar lavage fluid (BALF). The lungs were subsequently flushed another eight times with 10 ml PBS to obtain AMs.

**Counting of alveolar macrophages**

As we described previously [27], BALF was resuspended in Dulbecco’s modified Eagle medium (DMEM, Gibco, USA), counted, and transferred to 24-well culture plates (BD, Franklin Lakes, NJ, USA). After incubation for 60 min at 37 °C in a 5% CO₂ atmosphere, cultures were washed with DMEM to remove nonadherent cells. The adherent cells were counted using a hemocytometer, viability was determined using a 0.2% trypan blue exclusion assay, and cell differentiation and aggregation were examined by counting 500 cells on a Wright-Giemsa-stained slide. These cultures also served as the source for analyzing protein expression in AMs.

**LDH cytotoxicity assay**

Lactate dehydrogenase (LDH) release in the culture supernatant was assessed by the LDH Cytotoxicity Assay Kit (Promega, USA) according to the manufacturer’s instructions and was calculated as the percentage of total LDH. The experiment was performed in triplicate.

**Flow cytometry**

AMs were isolated from BALF were incubated with Fc block before staining with
fluorescently labeled propidium iodide (Immuno Chemistry Technology, USA), active
caspase-1 FAM-YVAD-FMK (ImmunoChemistry Technology, USA), and F4/80 (eBioscience,
USA) according to the manufacturer’s instructions. F4/80-, fluorescently labeled active
caspase-, and propidium iodide–positive cells indicated macrophage pyroptosis [29]. Flow
cytometry analysis was conducted with a BD LSR2 flow cytometer (BD Biosciences). Raw
data were analyzed using FlowJo software (TreeStar Corporation, USA).

Measurement of oxidative stress and antioxidant enzymes

The products of oxidative Stress (reactive oxygen species (ROS), malondialdehyde (MDA),
15-F2t-Isoprostane) and the antioxidant enzymes (superoxide dismutase (SOD),
glutathione peroxidase (GSH-PX), catalase (CAT)) were measured. Lung tissues were
homogenized in 5 volumes of RIPA bufffer, and the supernatants were collected after
centrifugation (2000 rpm at 4°C for 10 minutes) [22]. The ROS, MDA, SOD, GSH-PX, CAT
activities were measured according to the manufacturer’s instructions of assay kits
(Nanjing Jiancheng Bioengineering Institute, China). Free 15-F2t-isoprostane was
measured according to the enzyme immunoassay kit (Cayman chemical, USA). The
absorbance from the enzymatic reaction was detected at 412 nm, and the values were
expressed as pg/g wet protein in tissue homogenates [26].

Western blot analysis

As we described previously [8, 27], the left lung tissues and AMs were homogenized in
RIPA buffer (Thermo Scientific, USA) containing protease inhibitor cocktail (Sigma, USA)
and phosphatase inhibitor cocktail (Roche Applied Science, USA). Homogenates were
centrifuged at 13,000 rpm at 4 °C for 20 min. The supernatant was collected as the total
proteins. The cytoplasmic and nuclear proteins were extracted by NE-PER nuclear and
cytoplasmic extraction reagents (Pierce Biotechnology, USA) according to the
manufacturer’s instructions. The protein concentration was determined by Pierce BCA protein assay kit (Pierce Biotechnology, USA). Twenty microgram proteins per lane were separated on a polyacrylamide gel. The proteins were then transferred onto a polyvinylidene difluoride membrane. The membranes were incubated with the following primary antibodies overnight at 4 °C: Keap1 antibodies (1 : 200, Santa Cruz, California, USA), Nrf-2 antibodies (1 : 200, Santa Cruz, California, USA), HO-1 antibodies (1 : 200, Santa Cruz, California, USA), rabbit polyclonal HMGB1 antibody (1:1000, Abcam, USA), mouse monoclonal β-Actin antibody (1:5000, Abcam, USA), rabbit polyclonal Lamin A antibody (1:1000, Abcam, USA). Protein bands were visualized using enhanced chemiluminescence (Pierce, USA). The protein band intensities were normalized to those of β-Actin or Lamin A. The results under various experimental conditions then were normalized by mean values of the corresponding control.

Immunofluorescent staining

The expression of GSDMD proteins in AMs was determined by immunofluorometric assay. AMs were cultured on glass coverslips for 24 h. Following reoxygenation 4 h, cells were fixed in 4% paraformaldehyde for at least 10 min, then washed three times with PBS. After permeabilizing with 0.5% Triton X-100, cells were blocked with 5% BSA for 60 min. Then the cells were incubated with the primary antibody overnight at 4 °C on shaking table: GSDMD antibody (Novus Biologicals, NBP2-33422, USA). The cells were exposed to the specific secondary antibody for 1 h at room temperature. Finally, the cell nuclei were stained by 4′,6-diamidino-2-phenylindole (DAPI, Solarbio, China). Cells were viewed under a fluorescence microscope (Olympus BX51).

Enzyme-linked immunosorbent assay

ELISA kits for measuring mice IL-1β (R&D Systems, USA), IL-6 (R&D Systems, USA), NF-κB
(Abcam, USA) were used to quantify the contents of these cytokines in the lung tissues according to the manufacturer’ instructions. The quantity of IL-1β, IL-6, NF-κB in the lung tissues were standardized to the protein contents.

Statistical analysis

Parametric results in normal distribution are presented as mean ± S.E.M. All data were analyzed by one-way analysis of variance followed by the Tukey test if the data were normally distributed or by one-way analysis of variance on ranks followed by the Tukey test if the data were not normally distributed. Differences were considered significant at P < 0.05 based on two-tailed hypothesis testing. All statistical analyses were performed with SigmaStat (Systat Software, Point Richmond, CA).

Results

rHMGB1 preconditioning ameliorated inflammatory response and lung tissues damage induced by lung I/R

To confirm whether rHMGB1 preconditioning plays a key role in LIRI, rHMGB1 was used at 2 h before surgery. As shown in Fig. 1A, the control group and rHMGB1 group had an basically intact alveolar structure with few inflammatory cell infiltration. The I/R group had significantly alveolar walls rupture, neutrophil and erythrocyte infiltration, interstitial edema. These morphological changes were obviously alleviated in rHMGB1 + I/R group. The pathophysiologic changes were also significantly shown in lung injury scores and lung tissues W/D ratios (Fig. 1B, 1C). The expressions of IL-1β, IL-6, NF-κB measured by ELISA showed that these cytokines in I/R group were significantly higher than those in the control group and rHMGB1 group. Compared to the I/R group, the expressions of IL-1β, IL-6, NF-κB in rHMGB1 + I/R group were significantly decreased (Fig. 1D, 1E, 1F). The results of western blot analysis in Fig. 1G and 1H showed that preconditioning with rHMGB1 could
significantly reduce HMGB1 expression induced by lung I/R. No difference was noted between control group and rHMGB1 group in these experiments. These results suggested that preconditioning with rHMGB1 significantly ameliorated I/R-induced inflammatory response and lung tissues damage.

**rHMGB1 preconditioning alleviated lung I/R-induced AMs pyroptosis**

Firstly, we counted the amount of AMs isolated from BALF. Compared with control group and rHMGB1 group, AMs in BALF had significantly increased in the I/R group. Preconditioning with rHMGB1 significantly reduced I/R-induced isolated AMs (Fig. 2A). Secondly, we determined whether lung I/R could induce AMs pyroptosis. Distinctive features of pyroptosis included plasma membrane rupture, LDH release, caspases activity, and GSDMD cleavage [11]. As shown in Fig. 2B, compared to control group and rHMGB1 group, the release of LDH in AMs markedly increased in the I/R group, and the high expression of LDH induced by lung I/R could be significantly decreased by rHMGB1 preconditioning. Also, the results of flow cytometry demonstrated that rHMGB1 preconditioning significantly reduced AMs pyroptosis induced by lung I/R (Fig. 2C, 2D). Furtherly, Immunofluorescence staining in Fig. 5B specifically demonstrated that the expressions of GSDMD protein in isolated AMs were significantly increased by lung I/R, and the response was ameliorated by rHMGB1 preconditioning. Together, these results suggested that rHMGB1 preconditioning could alleviate AMs recruitment and pyroptosis induced by lung I/R.

**rHMGB1 preconditioning inhibited oxidative stress and increased antioxidant enzyme levels in LIRI**

To determine the effects of rHMGB1 on the oxidation and antioxidation processes during lung I/R, the products of oxidative stress (ROS, MDA, 15-F2t-Isoprostane) and the
antioxidant enzymes (SOD, GSH-PX, CAT) were measured. As shown in Fig. 3A-3C, compared with the control group and rHMGB1 group, the levels of ROS, MDA, and 15-F2t-isoprostane were markedly increased in I/R group. The high expressions of ROS, MDA, and 15-F2t-isoprostane in I/R group were significantly inhibited in the rHMGB1 + I/R group. Meanwhile, compared with the control group and rHMGB1 group, the activities of antioxidant enzymes (SOD, GSH-PX, CAT) were markedly decreased in I/R group. The low expressions of antioxidant enzymes induced by lung I/R were significantly recovered by preconditioning with rHMGB1 (Fig. 3D-3F). These results suggested that pretreatment with rHMGB1 might inhibited oxidative stress and increased the activity of antioxidant enzymes during lung I/R.

rHMGB1 preconditioning mediated the activity of Keap1/Nrf-2/HO-1 Pathway in LIRI

The Keap1/Nrf-2/HO-1 pathway plays an important role in the oxidation and antioxidation processes and can induce an array of intracellular antioxidant reaction. The nuclear protein Keap1, Nrf-2 and cytosolic protein HO-1 were analyzed by Western blot. Compared with the control and rHMGB1 groups, the protein expression of nuclear Keap1 was upregulated, and the protein expression of nuclear Nrf-2 and cytosolic HO-1 were downregulated in the I/R group. Preconditioning with rHMGB1 could significantly inhibit the high expression of Keap1 and elevate the low expression of Nrf-2 and HO-1 during lung I/R (Figs. 4). These results suggested that pretreatment with rHMGB1 could mediated the activity of Keap1/Nrf-2/HO-1 Pathway in LIRI.

Inhibiting Keap1/Nrf-2/HO-1 pathway alleviated the protective effects of rHMGB1 preconditioning in LIRI

Brusatol (a Nrf-2 antagonist) was administrated to further confirm that the protective
effects of rHMGB1 in LIRI is dependent on Keap1/Nrf-2/HO-1 pathway. As shown in Fig. 5A-5C, brusatol administration significantly increased the expression of nuclear Keap1 protein and decreased the expression of nuclear Nrf-2 and cytosolic HO-1 protein. The results demonstrated that brusatol could inhibit the expression of Keap1/Nrf-2/HO-1 pathway. Figure 5D showed that the pathological changes (such as alveolar destruction, inflammatory cell and erythrocyte infiltration, alveoli septum thickening) were significantly exacerbated in the brusatol + I/R group. Compared with the rHMGB1 + I/R group, lung tissues pathological damage were aggravated in brusatol + rHMGB1 + I/R group. Consistent with lung morphological changes, lung injury score and lung W/D weight ratio reflected the same changes in groups. In addition, Fig. 5G-5I showed that the expressions of IL-1β, IL-6, NF-κB were significantly increased in the I/R group. These pro-inflammatory factors induced by lung I/R could be exacerbated by brusatol administration, and alleviated by rHMGB1 administration. Compared with the rHMGB1 + I/R group, the expressions of IL-1β, IL-6, NF-κB were aggravated in brusatol + rHMGB1 + I/R group. Together, these results suggested that the protective effects of rHMGB1 in LIRI were obviously suppressed by brusatol administration.

Inhibiting Keap1/Nrf-2/HO-1 pathway suppressed the antioxidant effects caused by rHMGB1 in LIRI

As for oxidative stress production (ROS, MDA, and 15-F2t-isoprostane), the I/R group showed increased expressions as compared to those observed in the control group. rHMGB1 administration could markedly alleviate the oxidative stress induced by lung I/R. However, brusatol administration could aggravate the oxidative stress induced by lung I/R. Also, the expressions of ROS, and MDA, 15-F2t-isoprostane in brusatol + rHMGB1 + I/R group were drastically increased compared with that seen in the rHMGB1 + I/R group.
(Fig. 6A-6C). In addition, the levels of antioxidant enzymes (SOD, GSH-PX, and CAT) were significantly decreased induced by lung I/R, these changes could be reversed by rHMGB1 pretreatment. Similarly, compared with the I/R + rHMGB1 group, brusatol administration drastically suppressed the activities of SOD, GSH-PX, and CAT in I/R + rHMGB1 + brusatol group (Fig. 6D-6F). These results demonstrated that inhibition of Keap1/Nrf-2/HO-1 pathway could block the antioxidant effects of rHMGB1 pretreatment in LIRI.

rHMGB1 preconditioning inhibited AMs pyroptosis via Keap1/Nrf-2/HO-1 Pathway in LIRI

Compared with control group, the amount of AMs isolated from BALF was significantly increased in the I/R group. Preconditioning with rHMGB1 significantly reduced I/R-induced isolated AMs. The effect of rHMGB1 could be inhibited by brusatol administration (Fig. 7A). Consistent with the changes of isolated AMs amount, LDH release from BALF showed the same trend in groups (Fig. 7B). Furtherly, the results of flow cytometry showed that rHMGB1 preconditioning significantly reduced AMs pyroptosis induced by lung I/R, and the effect of rHMGB1 could be suppressed by brusatol administration (Fig. 7C, 7D). Also, the results of immunofluorescence staining in Fig. 7E and 7F demonstrated that GSDMD protein in isolated AMs were significantly increased by lung I/R, and the response was ameliorated by rHMGB1 preconditioning. Compared with the rHMGB1 + I/R group, the expressions of GSDMD in brusatol + rHMGB1 + I/R group were drastically increased. Together, these results suggested that inhibition of Keap1/Nrf-2/HO-1 pathway could block the anti-pyroptosis effects of rHMGB1 pretreatment in LIRI.

Discussion

HMGB1 is a familiar cytokine that mediates proinflammation and tissue damage in multiple organ pathologies. Usually, HMGB1 is secreted by activated macrophages, necrotic or
damaged cells. When HMGB1 is released into the extracellular milieu, it functions as an early inflammatory mediator and induces local and systemic inflammatory responses [30]. Preconditioning is a special effect that a minor previous injury prepares the body to better fend against a larger subsequent injury and results in decreased the irreversible organ damage. The current study demonstrated that rHMGB1 preconditioning provides protection against lung damage induced by lung I/R. Our finding is consistent with the previous results in organs IR injury [17–19, 31]. Our research and others suggest that rHMGB1 preconditioning, as a prevention strategy, might have potential clinical significance. Furtherly, we sought to explored the protective mechanisms of rHMGB1 preconditioning in LIRI.

Macrophages are the resident immune cells within the body and play a vital role in innate immune response. AMs are the primary resident cells within lung and can be activated by DAMPs during the progress of LIRI. Macrophages pyroptosis plays an important role in innate immune defense against intracellular bacteria or DAMPs. Recent studies confirmed that the inflammasomes promote the activation of inflammatory caspases such as caspases-1, -4, -5, -11, which in turn leads to cleavage of GSDMD. Unlike other forms of cell death, GSDMD as an effector molecule of pyroptosis induces pore formation, membrane rupture, and IL-1β maturation [9-10]. Therefore, pyroptosis strikes a unstable balance between protective host-defense response and harmful explosive inflammatory response [11–12]. An uncontrolled and amount of cells pyroptosis can induce tissue damage. Targeting pyroptosis in macrophages may be a potential strategy for inhibiting inflammatory response and organ damage [13–14]. In the present study, we found that preconditioning with rHMGB1 could alleviate pyroptosis-related proteins expression induced by lung I/R. It is plausible that the decreased expression of AMs pyroptosis ameliorated inflammatory factors release and lung I/R injury.
Oxidative stress is one of the critical factors in the pathophysiological process of LIRI [32]. During I/R, macrophages, neutrophils and endothelial cells can produce ROS, which induces proinflammatory cytokines. In addition, the excess generation of ROS can also trigger a oxidative response to cellular membranes through lipid peroxidation, leading to cell death in lung I/R. Several studies reported that the products of lipid peroxidation, such as MDA and 15-F2t-isoprostane, were significantly increased in the model of lung I/R, and exacerbated the inflammatory response, pulmonary edema and tissues damage [22, 33]. On the other hand, a series of antioxidant enzymes, such as SOD, GSH-PX, and CAT, have the function of eliminating the reactive free radicals to maintain the balance of oxidative and antioxidative stress response. Suppressing the activities of antioxidant enzymes could further aggravate inflammatory response and tissues damage during lung I/R [22, 34]. Consistent with previous studies, lung I/R could increase the products of oxidative stress (ROS, MDA, 15-F2t-Isoprostane) and decrease the expression of antioxidant enzymes (SOD, GSH-PX, CAT). It is known that HMGB1 can mediate inflammation via multiple ways, and has the function of activating reactive oxygen species and induce oxidative stress [20, 21]. Interestingly, the current study showed that pretreatment with rHMGB1 could reverse the high expression of oxidative stress and the low expression of the antioxidant enzymes during lung I/R. In addition, recent study demonstrated that suppressing pyroptosis via the activation of the antioxidant enzymes could ameliorate I/R injury [26]. Therefore, it is reasonable to think that the protective effect of rHMGB1 preconditioning in LIRI is partly attributed to decrease the expression of HMGB1, inhibit the oxidative stress, and then suppress AMs pyroptosis and inflammatory factor release during lung I/R.

We furtherly sought to explore the possible signal pathway underlying the protective effect of rHMGB1 preconditioning in suppress oxidative stress and AMs pyroptosis during
LIRI. Nrf2 is a stress sensing genetic transcription factor and has the function of mediating cellular antioxidant responses in stressful conditions [35]. Nrf2 and Keap1 are bound together in normal quiescent cells. when suffering stimulation, the hinge region of Keap1 is modified and generate a conformational change, resulting in Nrf2 degrading. And then activated Nrf2 cause the production of HO-1 [36]. The antioxidant response of Keap1/Nrf2/HO-1 signal pathway is referred to as the primary cellular defense against the cytotoxic effects of oxidative stress [25, 37]. Recent studies showed that activation of Keap1/Nrf2/HO-1 pathway may protect against oxidative stress and pyroptosis in organ I/R injury [22, 25–26]. In the current study, we observed that the expression of nuclear Keap1 was upregulated, and the expression of nuclear Nrf-2 and cytosolic HO-1 were downregulated after lung I/R. Pretreatment with rHMGB1 could reversed the activity of Keap1/Nrf-2/HO-1 pathway in LIRI. Furthermore, we used brusatol (a Nrf-2 antagonist) to further investigate the role of Keap1/Nrf2/HO-1 in the protective effects of rHMGB1 during LIRI. The results showed that brusatol administration upregulated the expression of Keap1 and inhibited the expression of Nrf2 and HO-1. Mediating Keap1/Nrf2/HO-1 pathway through brusatol administration could suppress the antioxidant and anti-pyroptosis effects of rHMGB1 in LIRI. However, a limitation of this study is that the molecule mechanism of rHMGB1 involved in the regulation of Keap1/Nrf2/HO-1 remains unclear. Further research in which AMs transfected with siRNA plasmid of Keap1/Nrf-2/HO-1 in oxygen-glucose deprivation/recovery (OGD/R) vitro model needs to be conducted to verify the present study and explore the specific interaction between rHMGB1 and Keap1/Nrf2/HO-1

Conclusions

These results provides evidences that rHMGB1 preconditioning protects against LIRI through suppressing AMs pyroptosis. The mechanism is partially explained by inhibiting oxidative Stress and improving the activity of antioxidative enzymes via Keap1/Nrf-2/HO-1
pathway.

Abbreviations

LIIR lung ischemia-reperfusion injury; I/R: ischemia-reperfusion; ALI: acute lung injury;
ARDS: acute respiratory distress syndrome; PRRs: pattern recognition receptors;
AMs: alveolar macrophages; DAMPs: damage-associated molecular patterns;
GSDMD: gasdermin-D; HMGB1: high mobility group protein 1; rHMGB1: recombinant HMGB1;
Nrf2: NF-E2-related factor-2; Keap1: Kelch-like ECH associating protein 1; HO-1: heme-
oxidase 1; H&E: Hematoxylin and eosin; W/D: wet/dry; BALF: bronchoalveolar lavage fluid;
LDH: lactate dehydrogenase; ROS: reactive oxygen species; MDA: malondialdehyde;
SOD: superoxide dismutase; GSH-PX: glutathione peroxidase; CAT: catalase; OGD/R: oxygen-
glucose deprivation/recovery

Declarations

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Author contributions

Lin Fei: Study design, fund collection, samples detection, manuscript preparation.

Xiao Jingyuan, Liang Fangte: Data interpretation, experiment operation, samples detection.

Dai Huijun, Ye Liu: Data collection, animal model.

Jing Ren, Lin Jinyuan: Statistical analysis.

Pan linghui: Study design, fund collection, manuscript preparation.

Ethics approval and consent to participate
The protocol was approved by the ethics committee of the authors’ hospital, Approval Numbers CS2016(22). No commercial entities providing devices or equipments had a role in any aspect of this study. All animal studies were carried out in accordance with the animal’s guidelines of the University Institutional Animal Care and Use Committee.

Consent for publication
This study consists of animal data and is devoid of any human data, thus consent for publication is not applicable.

Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests
The authors declare that they have no conflict of interests.

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Figures
rHMGB1 preconditioning ameliorated tissues damage, inflammatory response and HMGB1 expression induced by lung I/R. (A) Morphological changes in Control, rHMGB1, I/R and rHMGB1+I/R groups by H&E staining (×200). (B) The scoring of lung injury. (C) Graphic presentation of the wet/dry ratios in lung tissues. (D) Graphic presentation of IL-1β abundance in lung tissues. (E) Graphic presentation of IL-6 abundance in lung tissues. (F) Graphic presentation of NF-κB abundance in lung tissues. (G) Representative Western blot images of HMGB1 in nuclear protein. (H) Graphic presentation of HMGB1 abundance in nuclear protein. (*P < 0.05, compared with control group, □P < 0.05, compared between I/R and rHMGB1+I/R group).
rHMGB1 preconditioning decreases I/R-induced AMs pyroptosis. (A) Isolated alveolar macrophages counts in BALF. (B) LDH release by Isolated alveolar macrophages in BALF. (C) Representative images of flow cytometry analysis for determining macrophage pyroptosis were shown: F4/80+ cells were gated and analyzed for fluorescently labeled active caspase (FLICA) and propidium iodide (PI). (D) Quantitative analysis of F4/80+FLICA+PI+ cells. (E) Representative images of immunolabelling for GSDMD protein from isolated alveolar macrophages in BALF. (F) Graphic presentation of GSDMD abundance in isolated alveolar macrophages. (*P < 0.05, compared with control group, #P < 0.05,
rHMGB1 preconditioning inhibited I/R-induced oxidative stress and restored the antioxidant enzyme levels. (A) ROS. (B) MDA. (C) 15-F2t-isoprostane. (D) SOD. (E) GSH-PX. (F) CAT. (*P < 0.05, compared with control group, □P < 0.05, compared between I/R and rHMGB1+I/R group).
rHMGB1 preconditioning mediated the activity of Keap1/Nrf-2/HO-1 Pathway in lung I/R mice model. (A) Representative Western blot images of nuclear Keap1 in lung tissues. (B) Graphic presentation of nuclear Keap1 abundance in lung tissues. (C) Representative Western blot images of nuclear Nrf-2 in lung tissues. (D) Graphic presentation of nuclear Nrf-2 abundance in lung tissues. (E) Representative Western blot images of cytosolic HO-1 in lung tissues. (F) Graphic presentation of cytosolic HO-1 abundance in lung tissues. (*P < 0.05, compared with control group, ▲P < 0.05, compared between I/R and rHMGB1+I/R group).
Inhibiting Keap1/Nrf-2/HO-1 pathway alleviated the protective effects of rHMGB1 preconditioning in LIRI. (A) Western blot of nclear Keap1 in lung tissues. (B) Western blot of nclear Nrf-2 in lung tissues. (C) Western blot of cytosolic HO-1 in lung tissues. (D) Morphological changes in groups by H&E staining (×200). (E) The scoring of lung injury. (F) Graphic presentation of the wet/dry ratios in lung tissues. (G) Graphic presentation of IL-1β abundance in lung tissues. (H) Graphic presentation of IL-6 abundance in lung tissues. (I) Graphic presentation of NF-κB abundance in lung tissues. (*P < 0.05, compared with control group, □P < 0.05, compared with I/R, ^ P < 0.05, compared between rHMGB1+I/R and brusatol+rHMGB1+I/R group).
Inhibiting Keap1/Nrf-2/HO-1 pathway suppressed the antioxidant effects of rHMGB1 preconditioning in LIRI. (A) ROS. (B) MDA. (C) 15-F2t-isoprostane. (D) SOD. (E) GSH-PX. (F) CAT. (*P < 0.05, compared with control group, ▲P < 0.05, compared with I/R, ^P < 0.05, compared between rHMGB1+I/R and brusatol+rHMGB1+I/R group).
rHMGB1 preconditioning inhibited AMs pyroptosis via Keap1/Nrf-2/HO-1 Pathway in LIRI. (A) Isolated alveolar macrophages counts in BALF. (B) LDH release by Isolated alveolar macrophages in BALF. (C) Representative images of flow cytometry analysis for determining macrophage pyroptosis were shown: F4/80+ cells were gated and analyzed for fluorescently labeled active caspase (FLICA) and propidium iodide (PI). (D) Quantitative analysis of F4/80+FLICA+PI+ cells. (E) Representative images of immunolabelling for GSDMD protein from isolated alveolar macrophages in BALF. (F) Graphic presentation of GSDMD abundance in isolated alveolar macrophages. (*P < 0.05, compared with control group, □P <
0.05, compared with I/R, $^\wedge P < 0.05$, compared between rHMGB1+I/R and brusatol+rHMGB1+I/R group).